Quantification and differentiation of egg quality as affected by hen age and storage time, with the aid of near infrared hyperspectral imaging

by

Ruben Badenhorst

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Agriculture Sciences

at
Stellenbosch University
Animal Science, Faculty of AgriSciences

Supervisor: Dr Elsje Pieterse
Co-supervisor: Dr Janine Colling

March 2021

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2021

Summary

The aim of the study was to investigate the effect of hen age and egg storage time on certain internal, external and proximate egg quality parameters. Additionally, near infrared (NIR) hyperspectral imaging was used, in combination with multivariate data analysis, for the differentiation and quantification of egg quality as influenced by hen age as well as storage time. Amberlink hen eggs (n = 480) were collected from eight different age groups, which included ages 21, 29, 37, 45, 53, 61, 69 and 77 weeks. Each age group consisted of 60 eggs, which was further divided into six groups with 10 eggs per group, representing six different storage intervals. Storage days included 0 (day eggs were laid and collected), 15, 30, 45, 60 and 90 days where temperature was kept at roughly 15˚C and humidity at 75%. Eggs were first subjected to hyperspectral imaging, followed by physical quality analysis as well as proximate analysis, on the given storage days, to assess several egg quality parameters. The objectives for this study included: (i) to establish the effect of hen age on egg quality parameters; (ii) to study the effect of hen age and increased storage time on egg quality parameters and (iii) to investigate the application of NIR hyperspectral imaging for differentiation as well as quantification of hen egg quality. Hen age had a significant impact on the majority of the internal, external and proximate quality parameters. The most pronounced differences in egg quality parameters were observed in eggs collected from hens between the ages of 21 and 29 weeks. A significant interaction (hen age × storage time) for a few quality parameters were observed, while the rest of the parameters were either influenced by the main effect (storage time) or not affected. Eminent changes already occurred within the first 15 days of storage for the majority of the parameters. Near infrared hyperspectralimages for all eggs were acquired with a HySpex SWIR-384 pushbroom imaging system in the 400 – 2500 nm spectral range. Principal component analysis (PCA) could accurately separate eggs laid by different aged hens and stored for different time periods, and the trends observed correspond to differences in the proximate content. Subsequently, partial least squares regression (PLSR) was applied to the full dataset to construct quantitative prediction models for the Haugh unit (HU), protein, lipid and moisture content. Several optimization steps were implemented to test if the prediction models improved. This included, comparing the use of whole egg spectra with a smaller region of interest and a reduction in the wavelength. All constructed models showed reasonable accuracies, however refinement of the models is still needed. Overall the results indicated that hen age and storage time are two highly influential factors, which need to be considered when assessing egg quality. Hyperspectral imaging could also be used as a rapid, non-destructive screening technique for the successful differentiation and quantification of egg quality.

Opsomming

Die doel van die studie was om die effek van hen ouderdom en eier stoor tydperk op sekere interne, eksterne en chemiese eierkwaliteitparameters te ondersoek. Gevolglik was naby-infrarooi (NIR) hiperspektrale beelding gebruik, in kombinasie met meerveranderlike data-analise, vir die differensiasie en kwantifisering van eierkwaliteit, soos beïnvloed deur hen ouderdom sowel as stoor tydperk. Amberlink hen eiers (n = 480) is versamel uit agt verskillende ouderdomsgroepe wat ouderdomme 21, 29, 37, 45, 53, 61, 69 en 77 weke insluit. Elke ouderdomsgroep het 60 eiers bevat, wat verder in ses groepe verdeel is met 10 eiers per groep. Die ses groepe het ses verskillende stoor periodes verteenwoordig. Stoor periodes het 0 (dag wat die eier gelê en versamel is), 15, 30, 45, 60 en 90 stoor dae verteenwoordig, waar temperatuur by rofweg 15˚C and humiditeit by 75% gehou is. Eiers is eers geskandeer met hiperspektrale beelding, gevolg deur fisiese kwaliteitsanalise en chemiese analise, op die gegewe stoor periodes om verskeie eierskwaliteit parameters te beoordeel. Die doelstellings vir hierdie studie was: (i) om die effek van hen ouderdom op sekere eierskwaliteitparameters vas te stel; (ii) om die effek van hen ouderdom en stoor periode op sekere eierkwaliteitparameters te bestudeer en (iii) om die toepassing van NIR hiperspektrale beelding vir die differensiasie sowel as kwantifisering van eierkwaliteit te ondersoek. Hen ouderdom het 'n beduidende impak gehad op die meeste interne, eksterne en chemiese kwaliteit parameters. Die mees opvallende verskille, in die meerderheid eierkwaliteitparameters is waargeneem in eiers wat gelê was deur henne tussen die ouderdom van 21 en 29 weke. 'n Beduidende interaksie (hen ouderdom × stoor tydperk) vir enkele kwaliteitparameters is waargeneem, terwyl die res van die parameters beïnvloed is deur of die hoofeffek (stoor tydperk) of is glad nie beïnvloed nie. Noemingswaardige veranderinge het reeds binne die eerste 15 dae van stoor plaasgevind, vir die meerderheid parameters. Naby infrarooi hiperspektrale beelding is gebruik om te onderskei tussen eiers wat gelê was deur verskillende ouderdom henne en eiers wat vir verskillende periodes gestoor was. Beelde is verkry met 'n HySpex SWIR-384 stelsel in die 400 - 2500 nm spektrale reeks. Hoofkomponentanalise (HKA) kon eiers wat deur verskillende ouderdom henne gelê is en vir verskillende periodes gestoor akkuraat skei. Die resultate het ooreengestem met die chemiese inhoud. Gevolglik is gedeeltelike kleinste kwadraat regressie (GKKR) toegepas op die volledige datastel om kwantitatiewe voorspellingsmodelle vir die Haugh-eenheid (HU), proteïen, lipied en voginhoud te bou. Verskeie optimaliseringstappe is geïmplementeer om te toets of die voorspellingsmodelle sou verbeter. Heel-eierspektra is met 'n kleiner belangstellingsarea vergelyk en die golflengte is ook verminder. Alle modelle het aanvaarbare akkuraatheid getoon, maar verbetering aan die modelle is steeds nodig. Oor die algemeen het die resultate aangedui dat hen ouderdom en stoor tydperk twee invloedryke faktore is om in ag te neem wanneer eierkwaliteit beoordeel word. Hyperspektrale beelding kan ook gebruik word as 'n vinnige, nie-vernietigende tegniek vir suksesvolle differensiasie en kwantifisering van eierkwaliteit.

Acknowledgements

I wish to extend my sincere gratitude towards the following people and institutions, without them the completion of this thesis would not have been possible:

- I would like to thank God for giving me this incredible opportunity and being at my side every step of the way. With his guidance I was able to overcome arduous times and become stronger.
- To my parents Hein and Jolinda Badenhorst, who helped shape me into the man I am today. Due to their sacrifice I was not only able to study for a BSc degree but now for a MSc degree as well. They taught me that through diligent work as well as perseverance you can achieve anything and to never give up, no matter what. Thank you for all your support.
- To my girlfriend, Isél-Mari de Kock, for all her love and support, even at times when I wanted to quite or was not in a great mood. She contentiously believed in me and was always able to cheer me up. Thank you for standing by my side.
- A special thank you to both my supervisors Dr. E. Pieterse and Dr. J. Colling for all their support and guidance throughout my trials and thesis.
- I extend my gratitude to the Faculty of AgriScience of Stellenbosch and to Prof. L.C. Hoffman for their financial support, especially to tannie Adele for organizing this. Without them it would not have been possible to continue my research.
- To Rosendal Poultry for suppling the samples needed for this trial.
- I would like to specially thank Mr. Danie Bekker for not only his support and guidance but for his friendship as well. Thank you for all the laughs, coffee breaks and conversations. You are a great friend.
- I wish to thank the technical team at the Animal Science Department. To tannie Beverley for all her assistance with my analyses in the lab and friendship. Also, to Michael Mlambo, Janine Booyse and Jonas Christiaans, for their extremely long hours of assistance in the lab.
- I thank Professor M. Kidd for his assistance with my data analysis.
- Lastly, but not the least, I say thank you to all my family, friends and fellow students for your support and advice.

Notes

The language and style used in this thesis is in accordance with the *South African Journal of Animal Science,* with changes to increase readability. This thesis represents a compilation of manuscripts, where each chapter is an individual entity; thus, some repetition between chapters has been unavoidable.

Table of Contents

List of Tables

List of Figures

List of Equations

Abbreviations

3D	Three dimensional
a*	Redness
AOTF	Acousto-optic tunable filters
b*	Yellowness
Ca^{2+}	Calcium
$CaCO_3$	Calcium carbonate
CCD	Charge couple device
cm	Centimetre
CMOS	Complementary metal oxide semiconductor
CO_2	Carbon dioxide
CP	Crude protein
dist	Distilled
DM	Dry matter
EFSA	European Food Safety Authority
FOV	Field of view
FT-NIR	Fourier transform near-infrared
H_2O	Water
HCl	Hydrogen chloride
HDL	High density lipoproteins
HIA	Hyperspectral imaging analysis
HIS	Hyperspectral imaging
hr	Hours
HU	Haugh unit
Hz	Hertz
InGaAs	Indium gallium arsenide
K	Kelvin
L*	Lightness
LCTF	Liquid crystal tunable filters
LDA	Linear discriminant analysis
LDL	Low density lipoprotein
LED	Light emitting diodes
LSD	Least significance
MC	Mean centering

MCT	Mercury-cadmium-telluride
MDA	Multivariate data analysis
Mg^{2+}	Magnesium
MIA	Multivariate image analysis
min	Minutes
mL	Millilitre
MLR	Multiple linear regression
ms	Milliseconds
MSC	Multiple scatter correction
N	Nitrogen
NEO	Norsk electro optikk
NIR	Near-infrared
nm	Nano meter
OH	Hydroxide
P	Phosphorus
PbS	Lead sulphide
PC	Principal component
PCA	Principal component analysis
PCR	Principal component regression
PGP	Prism-grating-prism
PLS-DA	Partial least square discriminant analysis
PLSR	Partial least square regression
PRESS	Predictive residual error sum of squares
pt	Point
R^2	Coefficient of determination
RMSECV	Root mean square error of cross- validation
RMSEP	Root mean square error of prediction
ROI	Region of interest
RPD	Ratio of prediction to standard deviation
SD	Standard deviation
SG1D2P	Savitzky-Golay 1^{st} Derivative 2^{nd} Polynomial
SG2D3P	Savitzky-Golay, 2^{nd} Derivative, 3^{rd} Polynomial
SIMCA	Soft independent modelling of class analogy
SNV	Standard normal variate
SWIR	Short wave infrared

UV	Ultraviolet
Vis-NIR	Visible-near infrared
vs	Versus

Chapter 1
General Introduction

Hen eggs are one of the most essential protein sources of animal origin not only in South Africa, but also in numerous other countries as it forms part of many individuals' diets (Scanes, 2007). The global production of eggs has increased rapidly over the years, which reflects consumers' preference for more high-quality produced eggs and due to lower prices because of more effective production. Between 1995 and 2005 the production, and hence consumption, of chicken eggs has increased by 39%, a 16.9 million metric ton increase over a ten year period (FAO, 2007) indicating the vital role eggs play in the agricultural sector. Eggs are a source of many indispensable nutrients such as essential amino acids, omega-3 fatty acids, vitamins, minerals and much more (Sim, 1998; Sobamiwa, 1998; Sparks, 2005). Eggs are also one of the only food forms of animal origin that acts as a natural storage unit, protecting their inner content and quality for a certain duration (Grashorn, 2016). The decrease in egg quality can be caused by numerous factors and should therefore be considered when assessing egg quality to avoid wastage.

Factors affecting egg quality can include bird strain (Curtis *et al.*, 2005), nutrition (Bouvarel *et al.*, 2011), diseases (Charlton *et al.*, 2000), heat stress (Wolfenson *et al.*, 1981), temperature (Kirunda & McKee, 2000), humidity (Daniel & Balnave, 1981) and various other factors. However, hen age and storage time are some of the most important factors that influences egg quality and can have substantial effects on the internal and external egg quality characteristics (Stadelman & Cotterill, 1977; Chung & Lee, 2014). Increased hen age leads to an increase in the total egg weight with a proportional increase in the yolk (%) while the portion (%) of albumen decreases (Suk & Park, 2001). Increased storage time on the other hand can lead to a decrease in total egg weight, but also shows an increase in yolk (%) and decrease in the albumen (%) (Tilki & Saatci, 2004). The albumen quality can be influenced by these two factors as well, and is mainly determined by the Haugh unit (HU), which is directly related to the thick albumen height (Williams, 1992). The yolk is also influenced, where yolk quality is mostly determined by the colour of the yolk (Silversides & Scott, 2001) and the vitelline membrane strength (Beardsworth & Hernandez, 2004) surrounding the yolk. The eggshell quality consist mainly of the shell weight and the thickness (Robert, 2004), which is influenced by hen age (Britton, 1977), but is mostly unaffected by prolonged storage (Monira *et al.*, 2003; Jin *et al.*, 2011). However, Grashorn (2016) reported that egg shell quality was significantly affected by storage period. The protein and lipid content are fundamental components for consumers, as it can have direct health implications and can thus influence egg quality as well as egg safety (Attia *et al.*, 2014). Egg moisture is also considered an important constituent with regards to quality, since it can affect the albumen quality of eggs (Williams, 1992). It has been reported previously that the moisture, protein and lipid contents are influenced by hen age (Ahn *et al.*, 1997) and storage time (Omana *et al.*, 2011; Pereira *et al.*, 2011). It is thus crucial that these parameters

are also measured to determine egg quality and egg safety (eggs safe for consumption). Measuring egg quality has been implemented for the past century, but these measuring techniques present numerous drawbacks.

Egg quality testing plays a crucial role in the food industry to ensure eggs that are safe for consumption. It is thus necessary to investigate rapid alternative methods to replace the conventional testing methods currently used during manufacturing and processing. Current methods employed by the egg industry mostly include measurement of internal and external quality, with egg weight being the most common criteria used for grading. External egg quality is usually visually inspected by trained personnel for defects such as shell bumps and shell pigmentations. Cracks can be identified by using a method called candling (Weichman *et al.*, 1997). Determining internal egg quality poses a bigger problem due to its destructive procedure when compared to determining external egg quality, which causes little to no damage to the eggs during measurement. Internal quality is mostly determined by the characteristics of the albumen (Heiman & Carver, 1936; Jacob *et al.*, 2000). The albumen height along with the total egg weight is used to calculate the HU, which is used to determine internal egg quality (Haugh, 1937). All the egg proximate constituents should be considered when determining egg quality which include the protein, lipid and moisture content. However, the current methods used by the egg industry is time consuming, tedious, expensive and can lead to the destruction of eggs which contributes to wastage. There is thus a need for a rapid, non-destructive and cost-effective method to distinguish between eggs of different quality to ensure that it adheres to egg safety regulations. This can be achieved by using the near infrared (NIR) hyperspectral imaging technique.

Hyperspectral imaging is a technique that enables the user to rapidly take sample measurements. This imaging technique combines spectroscopy with digital imaging (Koehler *et al.*, 2002; Grahn & Geladi, 2007; Kamruzzaman *et al.*, 2013), which provides spectral and spatial information about samples (Reich, 2005; Wang & Paliwal, 2007; Amigo *et al.*, 2013). This is done by producing a three dimensional (3D) dataset, also known as a hypercube, containing two spatial dimensions (x and y) and one spectral dimension (λ) (Burger & Geladi, 2006). The hypercube is created when hundreds of single greyscale images are superimposed, where the greyscale images consist of various contiguous wavelengths within the NIR region (750 to 2500 nm) (Geladi *et al.*, 2004; Kumar & Mittal, 2010). The sample image consists of pixels each with its own unique spectrum, which could be used to determine the chemical composition of the analysed sample, enabling the user to classify components within the sample if prior knowledge is known about the sample (Burger & Geladi, 2006). Characteristics of the sample are obtained using multivariate image analysis (MIA) (Geladi *et al.*, 1989; Prats-Montalbán *et al.*, 2011) and if accurately analysed, it could be more reliable than previous imaging techniques (Menesatti *et al.*, 2010; Shao *et al.*, 2010).

Numerous studies have been done on the effect of hen age and storage time on the quality of eggs (Silversides & Scott, 2001; Tona *et al.*, 2004; Travel *et al.*, 2011; Akter *et al.*, 2014; Grashorn, 2016). Despite that, little research has been done on the interaction effect between these two factors

and if they influence egg quality differently. Studies have also been conducted on eggs using hyperspectral imaging. A Near infrared hyperspectral imaging study has demonstrated potential in determining egg quality as affected by storage (Suktanarak & Teerachaichayut, 2017), while other studies used conventional spectroscopic techniques (Kemps *et al.*, 2006; Giunchi *et al.*, 2008; Abdel-Nour *et al.*, 2011; Zhang *et al.*, 2015; Coronel-Reyes *et al.*, 2018). However, none of these studies used a storage duration longer than 42 days and none tried to distinguish and measure the protein, lipid and moisture content of eggs stored at different time intervals. These studies also did not test if it is possible to distinguish and measure the HU, protein, lipid and the moisture content of eggs laid by different aged hens. There is thus a need to expand on the knowledge of egg quality investigated in previous studies.

The aim of the study was to use NIR hyperspectral imaging, in conjunction with MIA for differentiation and quantification of egg quality as influenced by hen age and storage time. Specific objectives were therefore established to investigate:

- the effect of hen age on egg quality parameters,
- the effect of hen age with increased storage time on egg quality parameters and
- utilizing the information gathered from above-mentioned objectives to investigate egg quality using NIR hyperspectral imaging to a) distinguish between eggs laid by different aged hens and eggs stored for different time intervals with the aid of principal component analysis (PCA) as well as to b) construct prediction models with the aid of partial least squares regression (PLSR).

1.1 References

Abdel-Nour, N., Ngadi, M., Prasher, S. & Karimi, Y. (2011) 'Prediction of egg freshness and albumen quality using visible/near infrared spectroscopy', food bioprocess technol., 4(5), pp. 731–736.

Ahn, D. U., Kim, S. M. & Shu, H. (1997) 'Effect of egg size and strain and age of hens on the solids content of chicken eggs', Poult. Sci., 76(6), pp. 914–919.

Akter, Y., Kasim, A., Omar, H. & Sazili, A. Q. (2014) 'Effect of storage time and temperature on the quality characteristics of chicken eggs', J. Food, Agric. Environ., 12(3–4), pp. 87–92.

Amigo, J. M., Martí, I. & Gowen, A. (2013) 'Hyperspectral imaging and chemometrics. a perfect combination for the analysis of food structure, composition and quality', in Data Handl. Sci. Technol., pp. 344–366.

Attia, Y. A., Al-Harthi, M. A. & Shiboob, M. M. (2014) 'Evaluation of quality and nutrient contents of table eggs from different sources in the retail market', Ital. J. Anim. Sci., 13(2), pp. 369–376.

Beardsworth, P. M. & Hernandez, J.-M. (2004) 'Yolk colour - An important egg quality attribute', Int. Poult. Prod., 12(5), pp. 17–18.

Bouvarel, I., Nys, Y. & Lescoat, P. (2011) 'Hen nutrition for sustained egg quality', in Improv. Saf. Qual. Eggs Egg Prod. Egg Chem. Prod. Consum. Elsevier Inc., pp. 261–299.

Britton, W. M. (1977) 'Shell membranes of eggs differing in shell quality from young and old hens',

Poult. Sci., 56, pp. 647–653.

Burger, J. & Geladi, P. (2006) 'Hyperspectral NIR imaging for calibration and prediction: A comparison between image and spectrometer data for studying organic and biological samples', Analyst, 131(10), pp. 1152–1160.

Charlton, B., Bermudez, A., Boulianne, M., Halvorson, D., Jeffrey, J., Newman, L., Sander, J. & Wakenell, P. (2000) Avian disease manual, Am. Assoc. Avian Pathol. Pennsylvania, U.S.A.

Chung, S. H. & Lee, K. W. (2014) 'Effect of hen age, Storage duration and temperature on egg quality in laying hens', Int. J. Poult. Sci., 13(11), pp. 634–636.

Coronel-Reyes, J., Ramirez-Morales, I., Fernandez-Blanco, E., Rivero, D. & Pazos, A. (2018) 'Determination of egg storage time at room temperature using a low-cost NIR spectrometer and machine learning techniques', Comput. Electron. Agric. Elsevier, 145(December 2017), pp. 1–10.

Curtis, P. A., Kerth, L. K. & Anderson, K. E. (2005) Quality and compositional characteristics of layer hens as affected by bird age.

Daniel, M. & Balnave, D. (1981) 'Responses of laying hens to gradual and abrupt increases in ambient temperature and humidity', Aust. J. Exp. Agric., 21(109), pp. 189–195.

FAO (2007) Food and Agricultural Organization (FAO) ProdSTAT: Livestock (primary and processed).

Geladi, P., Isaksson, H., Lindqvist, L., Wold, S. & Esbensen, K. (1989) 'Principal component analysis of multivariate images', Chemom. Intell. Lab. Syst., 5(3), pp. 209–220.

Geladi, P., Burger, J. & Lestander, T. (2004) 'Hyperspectral imaging: Calibration problems and solutions', in Chemom. Intell. Lab. Syst., pp. 209–217.

Giunchi, A., Berardinelli, A., Ragni, L., Fabbri, A. & Silaghi, F. A. (2008) 'Non-destructive freshness assessment of shell eggs using FT-NIR spectroscopy', J. Food Eng., 89(2), pp. 142–148.

Grahn, H. F. & Geladi, P. (2007) Techniques and applications of hyperspectral image analysis, tech. appl. hyperspectral image analysis.

Grashorn, M. (2016) 'Effects of storage conditions on egg quality', 50(May), pp. 23–25.

Haugh, R. . (1937) 'The Haugh unit of measuring egg quality', US Poult. Mag, 43, pp. 522–555, 572–573.

Heiman, V. & Carver, J. S. (1936) 'The albumen index as a physical measurement of observed egg quality', Poult. Sci., pp. 141–148.

Jacob, J. P., Miles, R. D. & Mather, F. Ben (2000) 'Egg Quality 1', Anim. Sci., pp. 1–12.

Jin, Y. H., Lee, K. T., Lee, W. I. & Han, Y. K. (2011) 'Effects of storage temperature and time on the quality of eggs from laying hens at peak production', Asian-Australasian J. Anim. Sci., 24(2), pp. 279–284.

Kamruzzaman, M., ElMasry, G., Sun, D. W. & Allen, P. (2013) 'Non-destructive assessment of instrumental and sensory tenderness of lamb meat using NIR hyperspectral imaging', Food Chem. Elsevier Ltd, 141(1), pp. 389–396.

Kemps, B. J., Bamelis, F. R., De Ketelaere, B., Mertens, K., Tona, K., Decuypere, E. M. & De Baerdemaeker, J. G. (2006) 'Visible transmission spectroscopy for the assessment of egg freshness', J. Sci. Food Agric., 86, pp. 1399–1406.

Kirunda, D. F. K. & McKee, S. R. (2000) 'Relating quality characteristics of aged eggs and fresh eggs to vitelline membrane strength as determined by a texture analyzer', Poult. Sci., 79(8), pp. 1189–1193.

Koehler, F. W., Lee, E., kidder, L. H. & Lewis, E. N. (2002) 'Near infrared spectroscopy: the practical chemical imaging solotion', Spectrosc. Eur., 14, pp. 12–19.

Kumar, S. & Mittal, G. S. (2010) 'Rapid detection of microorganisms using image processing parameters and neural network', Food Bioprocess Technol., 3(5), pp. 741–751.

Menesatti, P., Antonucci, F., Pallottino, F., Roccuzzo, G., Allegra, M., Stagno, F. & Intrigliolo, F. (2010) 'Estimation of plant nutritional status by Vis-NIR spectrophotometric analysis on orange leaves [Citrus sinensis (L) Osbeck cv Tarocco]', Biosyst. Eng., 105(4), pp. 448–454.

Monira, K. N., Salahuddin, M. & Miah, G. (2003) 'Effect of breed and holding period on egg quality characteristics of chicken', Int. J. Poult. Sci., 2(4), pp. 261–263.

Omana, D. A., Liang, Y., Kav, N. N. V. & Wu, J. (2011) 'Proteomic analysis of egg white proteins during storage', Proteomics, 11(1), pp. 144–153.

Pereira, A. L. F., Vidal, T. F., Abreu, V. K. G., Zapata, J. F. F. & Freitas, E. R. (2011) 'Type of dietary lipids and storing time on egg stability', Food Sci. Technol., 31(4), pp. 984–991.

Prats-Montalbán, J. M., de Juan, A. & Ferrer, A. (2011) 'Multivariate image analysis: A review with applications', Chemom. Intell. Lab. Syst., 107(1), pp. 1–23.

Reich, G. (2005) 'Near-infrared spectroscopy and imaging: Basic principles and pharmaceutical applications', Adv. Drug Deliv. Rev., 57(8), pp. 1109–1143.

Robert, J. R. (2004) 'Factors affecting egg internal quality and egg shell quality in laying hens', J. Poult. Sci., 41(3), pp. 161–177.

Scanes, C. G. (2007) 'The global importance of poultry', Poult. Sci., 86(6), pp. 1057–1058.

Shao, X., Bian, X., Liu, J., Zhang, M. & Cai, W. (2010) 'Multivariate calibration methods in near infrared spectroscopic analysis', Anal. Methods, 2(11), pp. 1662–1666.

Silversides, F. G. & Scott, T. A. (2001) 'Effect of storage and layer age on quality of eggs from two lines of hens', Poult. Sci., 80(8), pp. 1240–1245.

Sim, J. S. (1998) 'Designer eggs and their nutritional and functional significance. In: Simopoulos, A.P. (Ed.), The return of omega-3 fatty acids into the food supply, 1. land-based animal food products and their health effects', pp. 89–101.

Sobamiwa, O. (1998) 'Performance and egg quality of hens fed cocoa husk based diet ', Niger. J. Anim. Prod. , 25(1), pp. 22–24.

Sparks, N. H. C. (2005) The hen's egg-is its role in human nutrition changing?

Stadelman, W. J. & Cotterill, O. J. (1977) Egg science and technology.

Suk, Y. O. & Park, C. (2001) 'Effect of breed and age of hens on the yolk to albumen ratio in two

different genetic stocks', Poult. Sci., 80(7), pp. 855–858.

Suktanarak, S. & Teerachaichayut, S. (2017) 'Non-destructive quality assessment of hens' eggs using hyperspectral images', J. Food Eng. Elsevier Ltd, 215, pp. 97–103.

Tilki, M. & Saatci, M. (2004) 'Effects of storage time on external and internal characteristics in partridge (Alectoris graeca) eggs', Rev. Med. Vet. (Toulouse)., 155(11), pp. 561–564.

Tona, K., Onagbesan, O., De Ketelaere, B., Decuypere, E. & Bruggeman, V. (2004) 'Effects of age of broiler breeders and egg storage on egg quality, hatchability, chick quality, chick weight, and chick posthatch growth to forty-two days', J. Appl. Poult. Res., 13(1), pp. 10–18.

Travel, A., Nys, Y. & Bain, M. (2011) 'Effect of hen age, moult, laying environment and egg storage on egg quality', in Improv. Saf. Qual. Eggs Egg Prod. Egg Chem. Prod. Consum.

Wang, W. & Paliwal, J. (2007) 'Near-infrared spectroscopy and imaging in food quality and safety', Sens. Instrum. food qual. saf. springer science and business media LLC, 1(4), pp. 193–207.

Weichman, F. L., Schoot, J. & Kenway, D. J. (1997) Egg candling system.

Williams, K. C. (1992) 'Some factors affecting albumen quality with particular reference to Haugh unit score', Worlds. Poult. Sci. J., 48(1), pp. 5–16.

Wolfenson, D., Frei, Y. F., Snapir, N. & Berman, A. (1981) 'Heat stress effects on capillary blood flow and its redistribution in the laying hen', Pflügers Arch. Eur. J. Physiol. Springer-Verlag, 390(1), pp. 86–93.

Zhang, W., Pan, L., Tu, S., Zhan, G. & Tu, K. (2015) 'Non-destructive internal quality assessment of eggs using a synthesis of hyperspectral imaging and multivariate analysis', J. Food Eng. Elsevier Ltd, 157, pp. 41–48.

Chapter 2
Literature Review

2.1 Introduction

Hen eggs are an inexpensive animal protein source and is an essential staple food in many countries (Sobamiwa, 1998). Eggs are a source of many important nutrients that include essential amino acids, omega-3 fatty acids, selenium, lutein, vitamins D, E, B and minerals such as iron, copper and zinc, making the egg a functional food (Stadelman & Cotterill, 1977; Sim, 1998; Sparks, 2005). While it is possible to improve the nutrient content of eggs through animal nutrition to some extent, conventionally produced eggs still offer many benefits and can have a fundamental impact on human health (Owren *et al.,* 1964; Holman *et al.,* 1982; Song & Kerver, 2000; McNaughton & Marks, 2002). Eating eggs regularly can improve satiety, weight loss, prevent muscular degeneration, improve brain function and assist the nervous system with functioning more effectively (Bertechini & Mazzuco, 2013).

Although eggs may provide a rich source of numerous essential nutrients, it can easily succumb to a decrease in quality due to the influence of various factors. Hen age and storage duration are regarded as the most important contributing factors to egg quality degradation (Stadelman & Cotterill, 1977; Chung & Lee, 2014). A vast amount of research has been done on how egg quality characteristics can be improved and how it is influenced by external factors. It is thus important to be able to distinguish between eggs of different qualities, as it is the main factor that influences consumers' preference and the producers profitability (Bejaei *et al.,* 2011). Testing egg quality is also important to ensure that the product is safe for consumption and has no negative effect on human health. Consumption of low quality eggs can lead to pathogen exposure, such as *Salmonella* (Adesiyun *et al.,* 2005).

Various conventional methods for testing egg quality have been used in the industry for decades. However, these techniques which include chemical analysis and determination of the Haugh Unit (HU), discussed later in this chapter, proved to have numerous drawbacks. Examples include that methods can be time-consuming, labour intensive, tedious, expensive and leads to the destruction of the sample in order to obtain the results (Suktanarak & Teerachaichayut, 2017). Cross contamination can occur during handling of eggs when conventional testing methods are implemented leading to less accurate results. A more objective, non-destructive and rapid screening method is thus needed to ensure that low quality eggs do not enter the food chain. The application of a spectroscopic technique known as near infrared (NIR) hyperspectral imaging could be used as a solution.

Near infrared hyperspectral imaging combines conventional spectroscopy with imaging to study the chemical composition of samples known as chemical mapping (Burger & Geladi, 2006;

Woodcock *et al.,* 2008; Valous *et al.,* 2009). Near infrared hyperspectral imaging produces three dimensional datasets or hypercubes containing both the spectral and spatial information of a given sample. In contrast, conventional spectroscopic techniques only collects an average spectrum, which is representative of the entire sample (Gowen *et al.*, 2007; Qin, 2010). NIR spectroscopy involves the analysis of the reflection or absorption of radiation by a sample in the near infrared range from 750 to 2500 nm. The spectra along with chemical assessment and prior knowledge of the samples can be used for the quantitative and qualitative evaluation of the samples. This can be achieved by making use of multivariate data analysis tools.

This literature study aims to address the effect of different factors that influences egg quality. It also elaborates on conventional testing methods and provides background information about NIR spectroscopy, the application of NIR hyperspectral imaging and multivariate data analysis as an alternative, improved testing method for quality control.

2.2 Egg production and composition

Birds produce eggs through a complex series of steps. The number of eggs produced and egg weight are important to the industry as these attributes hold a direct connection to profitability. Egg productivity and weight can vary depending on the age of the hen. Hens reach maturity at the beginning of the laying cycle, which is normally between the ages of 16 and 24 weeks as displayed in Figure 2.1. Peak production occurs between the ages of 24 and 26 weeks, followed by a gradual decrease as the hen ages. The egg weight shows a sharp increase at a young age and increase moderately as the hen ages. Egg production is typically sustained up to 72 to 74 weeks of age. Some hens are still capable of producing eggs above this age, but for commercial farms it is not always feasible as it does not provide a sustainable income (Travel *et al.,* 2011).

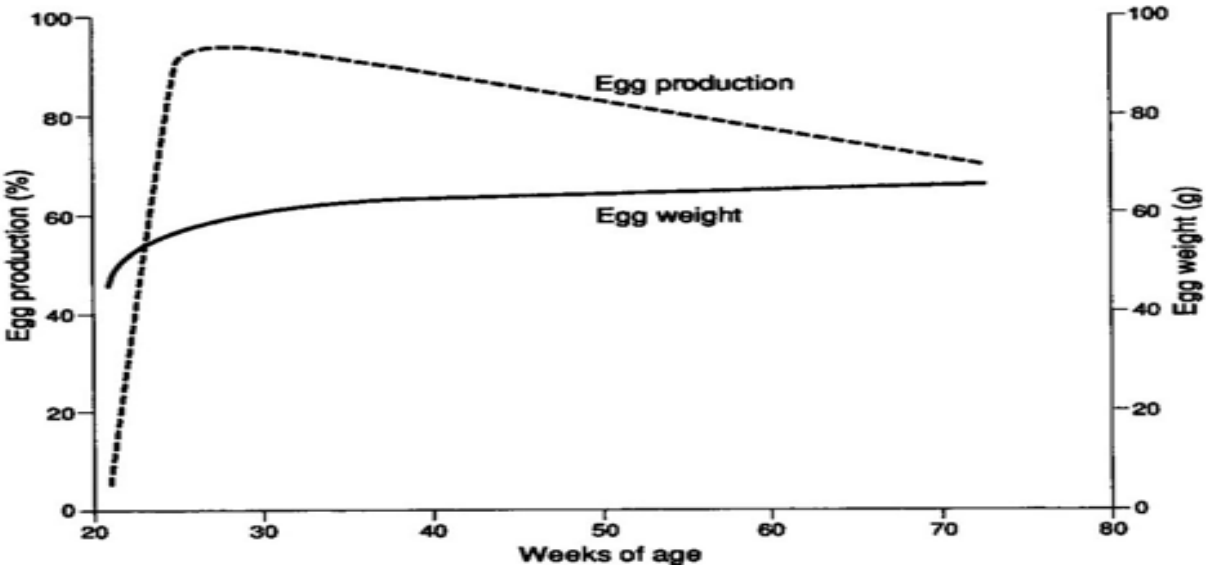


Figure 2.1 Typical production curve of a laying hen (Jacob *et al.*, 2003).

Egg quality depends on the internal and external characteristics of an egg. A standard egg consists of three major parts, each contributing a different percentage to the total composition. These include the internal albumen (58%), the yolk (31%) and the external shell (11%) (Stadelman & Cotterill, 1977). When only the internal contents are considered, the albumen constitutes 65% and the yolk 35% of the total contents. The eggshell as well as the eggshell membranes forms the outer part of the egg, which surrounds the albumen. The albumen surrounds the yolk, which is located at the centre of the egg (Rose-Martel *et al.,* 2012) (Figure 2.2). A thin layer known as the shell cuticle, along with two shell membranes and a calcium carbonate layer makes up the eggshell components, where the cuticle is the most external layer of the shell. The cuticle layer protects the egg against pathogens and prevents water loss through pore canals situated on the outer layer. It also serves as a temperature regulating mechanism, protecting the internal contents of the egg (Bruce & Drysdale, 1994; Tazawa & Whittow, 2000; De Reu *et al.*, 2006). The cuticle layer contains proteins, lipids and a small amount of carbohydrates (Rose-Martel *et al.,* 2012). Eggshell membranes consist of the inner membranes and outer membranes separated with an intertwined thread like structure, which also aids in prevention of pathogen invasion (Xiao *et al.,* 2014).

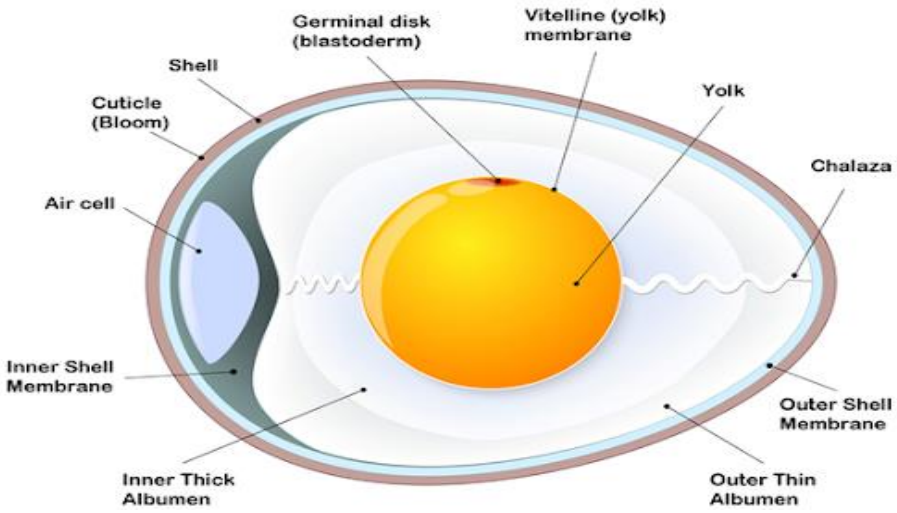


Figure 2.2 Graphic illustration of the internal and external content of a hen egg (Catinhat, 2016).

The egg albumen is a watery solution (roughly 85%) and the dry matter consist of 90% protein, 6% minerals and 0.8% carbohydrates, with 50% of the carbohydrates mainly occurring in the free form known as glucose (Mine, 1995; Sisaka *et al.*, 2006). The albumen serves to protect the embryo, and provides a source of protein, minerals and water during development. The albumen protein mainly consist of ovalbumin, ovomucoid, ovotransferrin, lysozyme, ovomucin and globulins, with the lysozyme-ovomucin complex being the influential proteins determining albumen quality (Toussant & Latshaw, 1999). The albumen consists of two major components or layers, known as the inner thick albumen (19.5%) surrounding the vitelline membrane and the outer thin albumen (80.5%) located next to the shell membrane (Powrie & Nakai, 1986) (Figure 2.2). The thick white spreads contain double the quantity of cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) compared to the thin

white spreads. It is believed that these cations play a role in maintaining the viscosity of the albumen (Cotteril *et al.,* 1992). The chalaziferous layer forms a twisted, gelatinous structure, attached at opposite sides of the vitelline membrane, while the other ends are connected to the outer thick white and the outer shell membrane (Burley & Vadehra, 1989). This structure, known as the chalaza, is responsible for protecting the yolk and keeping the germinal disc in an upward position to ensure that the embryo develops perpendicular (Kochav & Eyal-Giladi, 1971).

The egg yolk consist of 98% yellow yolk and approximately 2% white yolk (mostly protein) and is surrounded by the vitelline membrane (McNally, 1943). The vitelline membrane is the last defence boundary against microbes and also prevents the egg yolk and albumen from mixing (Mann & Mann, 2008). The egg yolk is a combination of triglyceride-rich lipoproteins, proteins and water in the form of an emulsion. A 100 g of egg yolk would supply 16% protein and more than 32% lipids. Lipids constitute a large component of the yolk and they are bound to proteins, forming lipoproteins. The lipoproteins can either be low density lipoprotein (LDL) or high density lipoproteins (HDL) (Moussa *et al.,* 2002). Attached to the yolk is the geminal disc, which gives rise to the embryo after fusing with spermatozoa (Patterson, 1909). A fertilized germinal disc is known as a blastoderm and a unfertilized one a blastodisc (O'Malley, 2005). These internal and external components of the egg can be affected by numerous factors. It is thus important to examine exactly what factors can lead to these quality changes in the structural components of the egg.

2.3 Egg quality

Eggs are affordable, nutritious and abundantly available. It is an excellent source of protein, essential vitamins and minerals (SAPA, 2017). Eggs can be of great value to help sustain the ever-growing population and human's requirement for more nutritional foods. However, the increasing consumers' demand over the past decades, has placed substantial pressure on the poultry industry to produce eggs of higher quality. Nutritionists and geneticists conducted a vast amount of research to enhance not only egg production, but quality as well, through breeding, management and optimising layer hen diets (Dunn-Horrocks *et al.,* 2011; Travel *et al.,* 2011).

2.3.1 Factors affecting egg quality

Egg quality, which include internal and external quality, is essential for profitability in the industry. Poor egg quality is an enormous problem in the commercial egg industry (Muir *et al.,* 1975). It is estimated that 1 billion eggs are wasted in the United States per year (Vogliano & Brown, 2016). This provides a clear indication of the financial incentive for the farmer and the industry to maintain a high egg quality for the consumer. The complex process of egg formation can lead to the internal or external egg quality being compromised at any stage and can be influenced by multiple factors. However, the most important factors to consider are hen age and storage duration (Stadelman & Cotterill, 1977).

2.3.1.1 Hen age

Over the years, hen age has proven to have a significant impact on the internal and external quality of eggs (Knox & Godfrey, 1937; Romanoff & Romanoff, 1949; Stadelman & Cotterill, 1977; Reis *et al.,* 1997; Robert, 2004). Egg weight might be the most important characteristic of eggs as it is the primary criterion used for grading and has an effect on profitability (Travel *et al.,* 2011). Egg weight increases as the hen ages as would be expected, which is also indicated by Figure 2.1. The increase in egg weight is due to changes in the different egg component proportions. While the shell weight stays relatively constant with increased age, the shell thickness decreases concomitantly (Roland, 1979). The decrease in thickness makes eggs more susceptible to cracks and breakage. On the other hand, the yolk to albumen weight ratio increases with increased hen age (Kaminska & Skraba, 1991). This is due to yolk and albumen weights' direct correlation to total egg weight as discussed in Chapter 3. Hen age also affects the egg shape, which includes egg height and diameter and the egg tends to become more elongated. Although the diameter also increases with an increase in hen age, it occurs at a slower rate than that recorded for the length (Travel *et al.,* 2011). Egg shell colour (brown eggs) and yolk colour can also be affected, becoming slightly paler as the hen ages. This is due to increased difficulty to absorb carotenoids (responsible for pigmentation) from the small intestinal track (jejunum) and incorporating the carotenoids into the shell and yolk (Surai *et al.*, 1999; Cherian, 2008).

The greatest influence of the hen's age is on the internal components of the egg (Romanoff & Romanoff, 1949). As mentioned before, hen age can lead to significant changes in the yolk and albumen solids (Kaminska & Skraba, 1991; Curtis *et al.,* 2005). The yolk height tends to increase drastically at a young age and continues to gradually increase, but the thick and thin albumen height slightly decreases as the hen gets older (Silversides & Scott, 2001; Bradley & King, 2016). Increase in the yolk height is due to the proportional increase in the egg weight. The thick and thin albumen height is at its highest on the day the eggs are laid. The thin albumen height decreased as well, but changes are not as pronounced as observed for the thick albumen height (Gennadios *et al.*, 1998; Curtis *et al.,* 2005). The gel like structure of the thick and thin albumen is dependent on the stability of the lysozyme with ovomucin complex. Increased hen age can lead to the disruption of disulphide bonds between lysozyme and ovomucin, which is covalently linked (Hammershøj & Qvist, 2001). Due to the increase in the yolk height and consequently the yolk weight, it is anticipated that the percentage of lipids will increase as the yolk contains a large majority of the total lipids present in an egg. This increase in yolk size will take up more space in the egg, therefore leading to a decrease in the amount of albumen available and thus less protein (%), as the albumen holds the majority protein present in the egg (Scott & Warren, 1941). There is thus an increase in the lipid to protein ratio with an increase in hen age (Flethcer *et al.*, 1983). This was confirmed by Ahn *et al.* (1997), reporting an increase in the solid content (protein and lipids) with a decrease in the moisture content with an increase in hen age.

The HU is the most widely used parameter to calculate the quality of an egg and has been implemented for the past century by scientists conducting egg quality studies. The HU is directly affected by the total egg weight and the thick albumen height (Chapter 3, Equation 3.2). An increase in hen age has a tendency to be associated with a decrease in HU due to the decrease in the thick albumen height as explained earlier (Haugh, 1937; Akbaş *et al.,* 1996; Ledur *et al.,* 2002; OnbaŞılar *et al.,* 2011).

2.3.1.2 Storage duration

The amount of time that an egg spends in storage is a factor that can have a large influence on egg quality, much like hen age. However, where an increase in hen age mostly leads to improved quality parameters such as increase in egg weight, an increase in storage duration has a more detrimental effect (Kamel *et al.,* 1980). There are several problems that can occur when eggs are stored for extended periods of time. The most significant impact is on the weight of the egg and albumen height (Stadelman & Cotterill, 1977). The reduction in weight during storage can be attributed to a loss of moisture from the albumen through the shell pores. For this reason, there are also changes in the albumen weight (Hill & Hall, 1980; Stadelman, 1986b). The colour of the yolk have a tendency to become paler as storage time increases due to disintegration of the carotenoids responsible for colour (Linden *et al.,* 1996). Prolonged storage results in a change in the yolk weight as well as the height. The yolk weight increases, while the height decreases. As storage duration increases the amount of carbon dioxide (CO_2) lost to the storage environment increases, resulting in an increase in albumen pH. The increase in pH is due to the conversion of the remaining carbon dioxide to carbonate. This reduction leads to a loss of structural integrity of the perivitelline membrane (Figure 2.2) that surrounds the yolk (Kirunda & McKee, 2000) and disrupts the osmosis equilibrium between the yolk and albumen (Dawes, 1975). This leads to the movement of water from the albumen to the yolk through means of osmosis. This increases the total amount of moisture in the yolk and leads to higher elasticity of the vitelline membrane, which contribute to the increase in the overall size of the yolk, but decreases yolk height (Brake *et al.,* 1997). It is evident that this movement of moisture from the albumen to the yolk will result in the reduction in not only the albumen weight, but the height as well. However, moisture is not only lost from the albumen to the yolk, but also lost from the albumen to the storage environment, culminating an exponential decrease in weight (Altan *et al.,* 1997; Tilki & Saatci, 2004). The longer eggs are stored, the greater the deterioration of the thick and thin albumen height (Figure 2.2). As storage time progress, the pH continues to increase and the isoelectric point of the lysozyme-ovomucin complex (9.2) is exceeded. This changes the stability of the lysozyme-ovomucin complex's disulphide bonds present in the thick and thin albumen resulting in the reduction in height and leads to liquefaction or albumen thinning (Omana *et al.*, 2011).

The percentage protein and lipids might also be affected due to a change in the yolk and albumen weight respectively, as it is already established that these attributes are correlated to each

other (Evans *et al.,* 1949, 1966). It has been reported that the protein and lipid content in stored eggs decrease due to increased protease activity (Omana *et al.*, 2011) and lipid oxidation (Akter *et al.*, 2014), respectively. As the HU is influenced by total egg weight and thick albumen height (section 2.2.2.1), it is evident that a decrease in both these factors will lead to subsequent decrease in the HU. An increase in storage time, therefore result in a decrease in egg quality (Miller, 2001; Samli *et al.,* 2005; Chung & Lee, 2014; Schoor *et al.,* 2017).

Increased storage durations can also lead to a decrease in shell weight and shell thickness, but studies have also shown that storage time has no significant impact on these characteristics (Monira *et al.,* 2003; Jin *et al.,* 2011). The decrease in the weight and thickness is a consequence of the shrinkage of the shell membranes and cuticle (Grashorn, 2016).

In summary, increase in storage duration causes a snowball effect in terms of reducing egg quality characteristics. The reason for this statement is that as storage duration increases, carbon dioxide and moisture is lost to the environment, increasing pH. This leads to the breakdown of albumen structure, perivitelline membrane and shell membranes, resulting in a reduction in total weight, yolk colour, protein (%), lipids (%) and most importantly HU. Therefore, the effect of storage time should be emphasized as it might be the single most important factor to consider when addressing egg quality. Nonetheless, there are other factors that can also have a noteworthy impact on egg quality characteristics.

2.3.1.3 Additional factors influencing egg quality

One of the factors that can have a substantial effect on egg quality is the strain of the bird. Genetic selection has led to new strains of birds with different production efficiencies, egg sizes during laying and even different shell quality characteristics (Curtis *et al.,* 1985). For example, differences in terms of egg size can be found between commercially selected layers and the common traditional layers (Hocking *et al.,* 2003). Although it is possible to select certain desirable traits, this can result in a weakening in the selection pressure of existing favourable traits (Falconer, 1954; Morris, 1985). It is important to monitor the selection program to safeguard all traits as most of them contribute to final egg quality (Poggenpoel *et al.,* 1996).

Studies have shown that between six to ten percent of all eggs produced have shell defects and most of these occurrences are a result of nutrition deficiencies (Washburn, 1982). Nutrition mainly affects the eggshell and not the internal components. Vitamins and minerals can play a big role in influencing external egg quality. Calcium may be one of the most important minerals required for shell formation since 95% of the shell consist of calcium carbonate ($CaCO_3$). If the birds have a phosphorous deficiency, this can cause this mineral to be extracted from the bird's skeletal structure and used for egg production (Leeson *et al.,* 1993). Vitamins such as vitamin C and E are known to reduce stress when supplemented in the diet and can in turn improve shell quality, while vitamin D can improve metabolism and absorption of calcium and phosphorous (Bollengier-Lee *et al.,* 1998; Brabin *et al.,* 2001; Selle & Ravindran, 2007). Water is just as important as nutrition as it can also

affect external egg quality. If water is supplied with high levels of electrolytes such as sodium and potassium, it could have detrimental effects on the eggshell (Balnave *et al.,* 1989). Providing cool drinking water can prevent heat stress and counter eggshell deteriorating as well as deformities (Xin *et al.,* 2002).

Heat stress, just like mycotoxins can result in reduced feed intake, leading to smaller eggs and decreased shell quality. If hens are subjected to high temperatures it can lead to the suppression of carbonic anhydrase enzyme activity, which is required for forming bicarbonate. Bicarbonate supply carbonate to the eggshell, which binds to calcium forming calcium carbonate the major component of eggshells (Wolfenson *et al.,* 1981).

Diseases can affect reproductive tissue leading to egg formation problems and reduced feed intake. This will lead to a decrease in egg production and malformations such as rough or soft-shelled eggs. Some diseases may also cause eggshells to become pale, wrinkled or even result in an irregular shape, which decreases exterior egg quality (Charlton *et al.,* 2000).

When it comes to the storage of eggs, there are two factors which can also play an important role to influence the quality of the egg and these include temperature and humidity. Increased temperature leads to a decrease in albumen quality. Temperature above 25˚C can result in a 50% decrease in HU and up to 48% decrease in the albumen height as well as a severe decrease in the strength of the vitelline membrane (Kirunda & McKee, 2000). Humidity does not influence the egg to the same extend as temperature, but can play a crucial role on the evaporation rate of moisture from the egg to the environment. This consequently affects the quality of the internal components (Funk, 1944; Daniel & Balnave, 1981). Most of these factors have an indirect effect on egg quality, nevertheless these factors are important to consider by the egg industry when undertaking improvement of egg quality.

2.4 Industry practices to evaluate egg quality

2.4.1 Egg grading

Grading is done by categorizing eggs by weight, quality, shape, shell and other factors. These attributes are important to evaluate the acceptability of the product for human consumption. Egg quality, as mentioned before depends on internal (yolk and albumen) as well as external (shell) quality. The South African egg industry produces a large range of egg types, but the two main colours are brown and white shelled eggs. The colour of the eggs are determined by heritability factors (Hall, 1944).

The most common criteria used for grading is egg weight. According to South African standards, egg size can range from small to super jumbo (Table 2.1). It is important to note that most industry practices do not weigh eggs individually, but rather in batches due to high egg loads reaching the factory.

Table 2.1 Classification of eggs based on weight, as stated by the Department of Agriculture, Forestry and Fisheries (DAFF, 1999)

Egg Size Description	Weight (g)
Super jumbo	≥ 72
Jumbo	≥ 66
Extra Large	≥ 59
Large	≥ 51
Medium	≥ 43
Small	≥ 33

There are several categories of shell defects or undesirable traits that should not be present on eggs. These include cracked, shell-less, soft-shelled, pimpled, banded, calcium deposits, white or brown speckled, blood stained, corrugated, misshaped and mottled eggs. The main contributing factors leading to these shell defects are hen age and nutrition. While characteristics such as bumps and pigments are graded by normal visual inspection by trained personnel, the cracks are identified by using a method called candling. This technique involves illuminating the eggs using a light. The light, which is transmitted through the egg, allows visualization of cracked eggs and other defects, as seen in Figure 2.3. Defects can also be identified by an electron crack detector (Weichman *et al.,* 1997; Pan *et al.,* 2005). These techniques offer rapid methods of evaluating shell parameters.

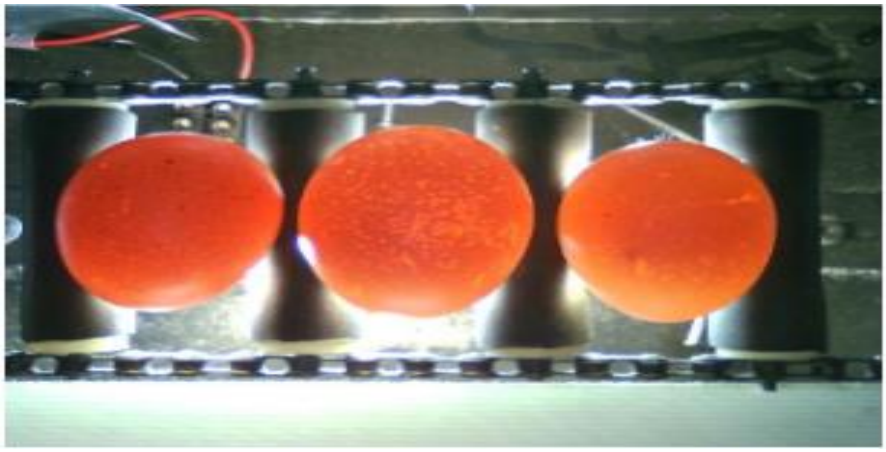


Figure 2.3 Egg-candling method used to detect egg defects (Vargas *et al*., 2018).

2.4.2 Conventional egg quality testing methods

Egg quality is the primary concern in the egg industry and for this reason suitable testing methods need to be implemented to ensure that the product is safe for consumption. There are various techniques used to determine egg quality. One of the major drawbacks of some of these testing methods are their destructive nature. Most of the conventional testing methods are based on measurement of the height, length, colour or gravimetrical measurements (Beardsworth & Hernandez, 2004; Kul & Seker, 2004; Karoui *et al.*, 2006).

Starting with the eggshell, quality measurements are mainly assessed through visualization by checking for cracks, bumps, pigments and other undesirable occurrences which are easy to identify

without damage to the samples. However, parameters such as shell weight and thickness can only be determined destructively through breaking the egg and removing internal components. The air cell of the egg can be used to give an indication of quality as well. Bigger air cells indicates higher loss in moisture, which is an indication of poor quality (Sharp & Powell, 1930). Air cell determination through candling is a non-destructive method, but can be time consuming and sometimes difficult to identify and grade (Weichman *et al.,* 1997).

None of the quality measurements done on the yolk can be performed without destructive consequences. The measurement of yolk quality attributes not only leads to wastage of the sample, but is time consuming as well. Yolk quality is based on the colour of the yolk and the strength of the vitelline membrane (Henderson, 1941). Yolk colour can be determined by using the Roche Yolk Colour Fan or a digital colour spectrophotometer (Vuilleumier, 1969; Beardsworth & Hernandez, 2004). The colour fan categorizes the yolk to a specific shade of colour ranging from bright yellow to dark orange. The digital colour spectrophotometer uses the Hunter colour scale principle (Figure 3.2) to categorize the yolk in terms of its lightness (L*), redness (a*) and yellowness (b*) (Hunter, 1958). Vitelline membrane strength or vitelline integrity is mostly judged on the rupturing occurrence of the yolk when broken out of its shell or when the yolk breaks if pressure is applied (Moran & Hale, 1935).

The most important component used to evaluate egg quality is the albumen (Heiman & Carver, 1936). The albumen height and albumen's spreading distance from the yolk can be used to determine egg quality. Both thick and thin albumen height is measured with a manual tripod micrometer, when the egg is broken out of its shell. As mentioned before, low thick and thin albumen heights indicate poor quality. Albumen spreading is used to assess the viscosity of the albumen (Cornforn *et al.,* 1969; Ibarz *et al.,* 1999). Eggs of poor quality have albumen with low viscosity, whilst eggs of higher quality have higher viscosity. The distance from which the thick and thin albumen move from the yolk can thus also be used as an internal quality indicator. The HU is possibly the most important measurement to determine egg quality. The HU is a measurement of albumen quality when an egg is broken out of its shell onto a flat surface (Haugh, 1937) and can be determined using Equation 3.2 (Chapter 3). Eggs can be classified into three groups based on their HU value as discussed in Section 3.2.2.

To determine total egg quality, one should consider the shell, yolk and albumen components. Analysis include determining the thick and thin albumen height, the albumen spreading distance as well as HU and the proximate analysis (protein, lipid and moisture content) of internal components. Measurements of these attributes are associated with a few drawbacks. In summary, they are often time consuming, expensive and lead to the destruction of the sample or a combination of these factors. There is thus a dire need for a rapid, non-destructive and a more economically viable screening method to differentiate not only between eggs of different qualities, but also their internal components to ensure increased egg quality for consumers. To meet this objective the application of a technique known as near infrared (NIR) hyperspectral imaging, which is based on the principles of NIR spectroscopy, was investigated.

2.5 Near infrared spectroscopy

Near infrared (NIR) spectroscopy is a technique that has been expanded and implemented more frequently over the past few decades and has offered several advantages for the food industry. In 1800, William Herschel was the first to discover near infrared light when he recorded an increase in temperature associated with the radiation from solar emission above red light (Herschel, 1800). After this in 1881, Abney and Festing (1881) recorded the spectral absorption/reflection of a variety of liquids in the spectral range between the 700 nm to 1200 nm. After studying the NIR spectra, they concluded that the hydrogen bonds involved in these liquids or other samples are of great importance when considering NIR absorption. It was later realised that functional groups absorb at different wavelengths and that this correlates to the band characteristics of C-H bonds in materials (Coblentz, 1905). After this, numerous studies of band assignment took place (Ellis, 1929; Bokobza, 1998; Pasquini, 2003; Sakudo *et al.*, 2006; Walsh & Kawano, 2009). However, the first application of NIR spectroscopy in the agricultural industry was by Hart *et al.* (1962), suggesting that the NIR instrumentation could be used as non-destructive method to determine protein and moisture content in grains.

Near infrared spectroscopy is a vibrational spectroscopic technique that utilizes photon energy in the 750 - 2500 nm part of the electromagnetic spectrum to gather information about samples (Osborne *et al.,* 1993; Pasquini, 2003; Walsh & Kawano, 2009). It is a non-destructive, fast technique used in various industries especially the food sector. NIR spectroscopy depends on the interaction between energy in electromagnetic radiation and chemical bonds in organic samples, such as C-H, S-H, N-H, and O-H (Miller, 2001). When a sample is exposed to the electromagnetic radiation it is possible for the radiation to be absorbed, transmitted or even reflected from the sample's surface (Scotter, 1997). This response can be measured and results in a NIR absorption or reflection spectrum. A spectrum consist of a diverse amount of absorption bands, and can provide chemical and/or structural information of a specimen (Osborne *et al.,* 1993; Cen & He, 2007). The absorption bands represent overtone bands, which arise from the fundamental vibrations of the covalent bonds (C-H, N-H, O-H and S-H) within a sample that absorb in the mid-infrared region. These bonds experience a change in energy when they are exposed to the electromagnetic radiation in NIR frequencies (Cen & He, 2007). There are multiple reactions or vibrational arrangements that can take place after these bonds absorb the energy in the near infrared radiation, but the most common occurrences include stretching and bending (Bokobza, 1998). Bending is the structural change in the bond angle between atoms, as seen in Figure 2.4a. Stretching on the other hand is explained as the change in the distance along the axis between atoms, as seen in Figure 2.4b (Workman, 1993). Absorption bands in spectra can be allocated to functional groups (e.g. C-H), which are typically found in compounds of interest such as protein, water, oil, etc. (Coblentz, 1905). In other words, molecular bonds start to vibrate when the energy of wavebands at a specific frequency correspond to the energy required for each type of molecular bonds to change its vibrational level. Therefore, the absorption of NIR radiation by various molecules in the sample is measured and a functional

group can be assigned to peaks. For example, water molecules (O-H bonds) absorb highly at 1450 nm (Osborne *et al.,* 1993), protein (N-H bonding group) at 1020 nm and oil (C-H bonding group) at 1151 nm (Ortiz-Somovilla *et al.,* 2007). A challenge with the interpretation of absorption bands is that NIR absorption bands are usually broad and overlap, as a result the absorbance at a given wavelength may correspond to various compounds since a compound can absorb at more than one wavelength (Menezes *et al.*, 2009; Williams *et al.*, 2019). This challenge can be overcome through the use of multivariate data analysis tools.

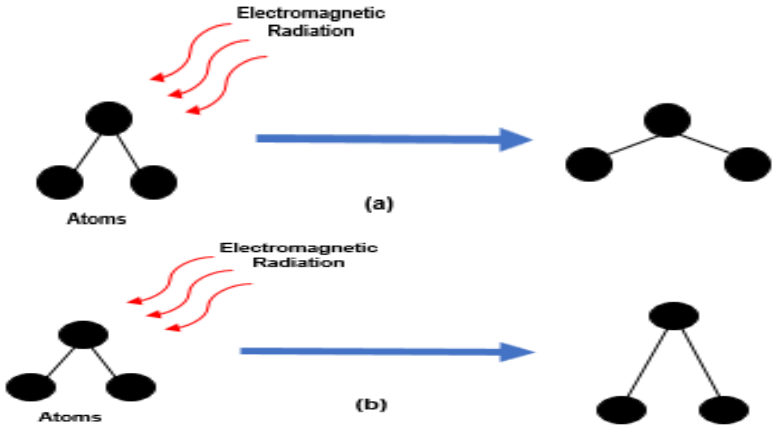


Figure 2.4 Illustration of how electromagnetic radiation can lead to the (**a**) bending and (**b**) stretching of covalent bonds between atoms.

As mentioned before, the popularity of NIR spectroscopy has increased over the years especially in the agricultural as well as the food industry. In the agricultural industries examples of its application include predicting the nutritional value (e.g. protein and starch) of wheat grain in broiler diets (Owens *et al.,* 2009). Cozzolino and Murray (2004) on the other hand used NIR spectroscopy to identify and authenticate different meat muscles in beef, lamb and chicken. In the food industry, NIR spectroscopy has been used to predict the fatty acid content of beef (Sierra *et al.,* 2008) and to detect undesirable substances in cereal (Vermeulen *et al.,* 2012). This technique has also shown value in the pharmaceutical and medical industry (Dyrby *et al.*, 2002; Blanco & Alcalá, 2006; Spahn *et al.*, 2008) for example examining lung injuries (Shibata *et al.*, 1999) and detecting certain compounds in tablets (Liu *et al.*, 2005).

NIR spectroscopy can be used for exploratory analysis to differentiate between samples or to determine similarities in terms of their chemical composition (e.g. protein content) or physical attributes (e.g. maize kernel hardness) (Cen & He, 2007). NIR spectroscopy can also be used to construct categorical or quantitative models that can be used to classify samples belonging to a specific group (e.g. samples derived or belonging to various species) or measure specific sample constitutes (e.g. quantify samples' specific protein content), respectively (ElMasry & Sun, 2010). However, to build quantitative models, the reference values of the samples need to be obtained through chemical analysis.

Although NIR spectroscopy offers numerous advantages there are also some drawbacks to the successful implementation of the technique.

2.5.1 Advantages and limitations of NIR spectroscopy

Some of the biggest advantages of using this method is that it offers a rapid, non-destructive technique to analyse samples. Samples need little or no preparation, which in turn saves time and NIR radiation allow for deeper penetration, with reduced photodamage (Chien *et al.*, 2018). Once a model exists, no reagents or preparation materials are required for analysis resulting in less costs and time spent on chemical analysis (Blanco & Villarroya, 2002; Pasquini, 2003). NIR methods can be developed to measure several chemical compounds simultaneously and make it possible to study properties such as particle size, viscosity and even density (Blanco & Villarroya, 2002). Although there are many benefits associated with this technique, there are also limitations.

One drawback of NIR spectroscopy is that developing models initially require investment in the chemical analysis of samples to prepare distinct calibration models (Osborne *et al.,* 1993). For example, to examine the freshness of eggs, physical analyses of the egg's HU need to take place before model preparation and construction. The spectra and chemical values are used to create a model and this stage can be time consuming and expensive at first (Suktanarak & Teerachaichayut, 2017). A large number of samples are required to ensure samples are representative of all the physical or chemical variation that may occur to develop the calibration model (Blanco & Villarroya, 2002). Developing models also require chemometric methods for data modelling to extract the relevant information (Blanco & Villarroya, 2002). Conventional spectroscopic instruments are limited in that it only captures an average spectrum of the sample, which is used to represent the entire sample. This limitation can be resolved by using hyperspectral imaging, which produces information on the spatial nature of samples (Goetz *et al.,* 1985; Feng *et al.,* 2013) and can be used to visualize how chemical compounds are distributed within the sample (Gowen *et al.,* 2007).

2.6 NIR Hyperspectral imaging

NIR hyperspectral imaging is a combination between spectroscopy and digital imaging (Koehler *et al.,* 2002; Burger & Geladi, 2006; Kamruzzaman *et al.,* 2013) and provides both spectral and spatial information of samples (Reich, 2005; Grahn & Geladi, 2007; Wang & Paliwal, 2007; Manley *et al.,* 2009; Amigo *et al.,* 2013). During imaging, the absorption over a spectral range (λ) is collected for every pixel with specific spatial coordinates (x and y) in the image (Burger & Geladi, 2006) as represented in Figure 2.5. This creates a three-dimensional dataset known as a "hypercube", which is created when a vast number of single greyscale images are superimposed, where the intensities of a single wavelength are represented by the greyscale images (Burger & Geladi, 2006; Kumar & Mittal, 2010). Each of these greyscale images also provide information about the sample and if accurately analysed, it could be more reliable than previous spectroscopy and imaging techniques (Menesatti *et al.,* 2010; Shao *et al.,* 2010).

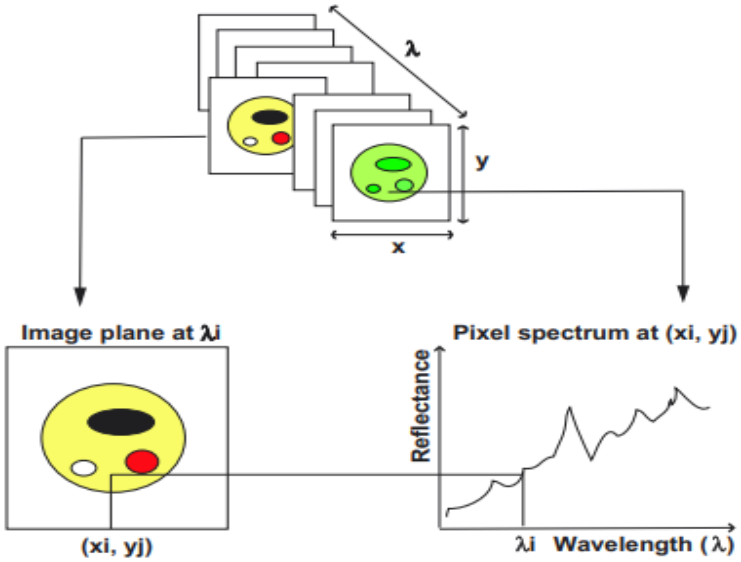


Figure 2.5 A simplified portrayal of a hypercube displaying the two spatial (x and y) and one spectral (λ) dimension (adapted from Gowen *et al.*, 2007).

NIR hyperspectral imaging was first applied as a technique to study the surface of the earth by using remote sensing (Goetz *et al.,* 1985). However, since the last 15 years the technology has developed and have been used in several industries, especially having a positive impact on the agricultural sector (Mehl *et al.,* 2004; Shahin & Symons, 2008; Wang *et al.,* 2016). Examples of successful studies using NIR hyperspectral imaging in the agricultural field include the exploration and classification of whole wheat kernels varying in hardness (Du Toit, 2009), identifying hard and soft endosperm in whole maize kernels (Williams *et al.,* 2009), predicting the anthocyanin content in wine grapes (Chen *et al.,* 2015) and a method to differentiate maize ear rot pathogens on growth media (Bezuidenhout *et al.,* 2018). Applications of NIR hyperspectral imaging is not only limited to the agriculture and food sectors, other fields such as the pharmaceutical and medical industry (Lyon *et al.*, 2002; Martin *et al.*, 2006; Amigo & Ravn, 2009; Tankeu *et al.*, 2016), remote sensing (Goetz *et al.,* 1985) and material science and industrial sectors have also benefitted from this technique (Tatzer *et al.,* 2005; Serranti *et al.,* 2012).

Similar to conventional spectroscopy, this technique offers fast analyses, little or no sample preparation, no physical contact and offers non-destructive analysis (Burger & Geladi, 2006; Gowen *et al.,* 2007). In contrast, NIR hyperspectral imaging provides the opportunity to view the degree of chemical or physical spatial heterogeneity within a specimen (Martinsen & Schaare, 1998). Differences in physical properties are caused by scattering, which can affect the degree of reflectance intensities (Gowen *et al.*, 2008b). It is thus essential to investigate the physical properties of the specimen before starting image analysis (McGoverin *et al.,* 2011). It is also important to know how the instrumentation works and how the images are obtained in order to fully understand the hyperspectral imaging process.

2.6.1 Instrumentation and image acquisition

The main components of a NIR hyperspectral imaging instrument include a detector, illumination source, sample stage, monochromator or spectrograph and camera, which are all connected to a computer or controlling software (Reich, 2005; Burger & Geladi, 2006; Wu & Sun, 2013).

2.6.1.1 Detector

The detectors record the light changes that take place and to do this wavelength modulators split broadband light to divide wavelengths, which make it possible to analyse each wavelength independently. Detectors used during hyperspectral imaging can be one of three types. These detectors include lead sulphide (PbS), Indium Gallium Arsenide (InGaAs) or Mercury-cadmium-telluride (MCT). These detectors are sensitive in the wavelength region of 1100 – 2600 nm (Boldrini *et al.,* 2012). The detectors which are sensitive to wavelengths between 400 – 1000 nm include the charge couple device (CCD) or a complementary metal oxide semiconductor (CMOS) (Gowen *et al.*, 2015).

2.6.1.2 Illumination source

The illumination unit supplies a light source, which illuminates the sample with NIR radiation. The illumination source can consist of xenon gas plasma lamps, tungsten halogen lamps, lasers or light emitting diodes (LED) (Wang & Paliwal, 2007; Qin *et al.,* 2013). It is not only the light source that is of importance, but also the orientation or configuration to obtain the image. The two main configurations of the illumination source is either transmittance or reflectance as seen in Figure 2.6 (Kim *et al.,* 2012). During transmittance spectroscopy the light source is positioned underneath the specimen and the light is passed through the sample towards the camera and detector. During reflectance spectroscopy the light source is placed above the specimen (usually at an angle) and reflected off of the sample surface in the direction of the camera and detector (Siska & Hurburgh, 1995; Burger & Geladi, 2006). Reflectance is measured as the amount of light reflected off the surface of the sample and is expressed as a fraction of the total light striking the surface. The same can be applied to transmitted light, which is the amount of light transmitted through the sample, again expressed as a fraction of the total amount of light striking the sample (Woolley, 1971).

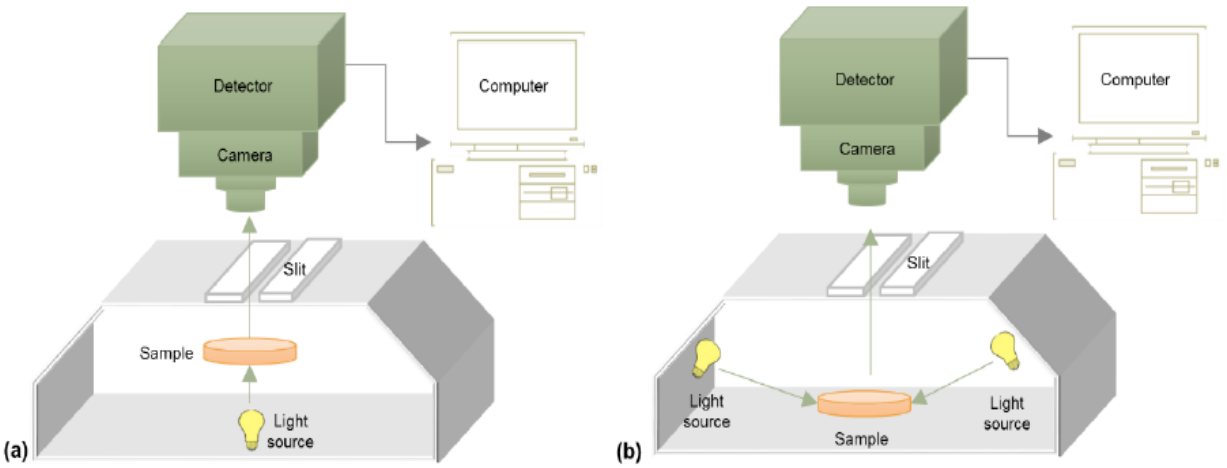


Figure 2.6 Near infrared hyperspectral imaging instrumentation where illumination source is positioned in (**a**) reflectance spectroscopy or (**b**) transmittance spectroscopy configuration (adapted from Bezuidenhout *et al.*, 2018).

Tungsten halogen lights are known as a source of broadband light. Using this light source in NIR imaging systems have numerous advantages, such as lower costs, extended spectral outputs and high intensities in the NIR range between 900 – 2500 nm (Gowen *et al.,* 2015). However, this light source tends to accumulate heat, which can be a problem when working with live organisms. It may also cause a drift in the spectral output and the operation period to use it is limited (Bergman & Parham, 1993). The xenon gas plasma lamps have desired aspects such as providing a stable continuous and more intense radiation in the visible and ultraviolet (UV) part of the spectrum (Anderson, 1951). The main disadvantage of the xenon gas lamp is that it can lead to overheating (Baum & Dunkelman, 1950). In hyperspectral imaging, fluorescence lasers can be used as an illumination source due to its ability to grant monochromatic light in a cavity filled with either dye, a semi-conductor or gas (Kim *et al.,* 2003). Light emitting diodes (LED's) can also be utilised as a source of light, which have the capability to cover a wide spectral range and are frequently used in the food industry (Gowen *et al.,* 2015). The reason for LED's popularity is due to its small size, low cost input, lower heat emittance and thus also an extension in lifespan (Gowen *et al.,* 2007; Chen *et al.,* 2010).

2.6.1.3 Spectrograph

The spectrograph or monochromator is used to separate and select wavelengths. To do this, one of three spectral coders are needed, namely liquid crystal tunable filters (LCTF), prism-grating-prism (PGP) or acousto-optic tunable filters (AOTF) (Burger & Geladi, 2006). The spectrograph may be the most important component of the hyperspectral imaging system. The reason for this is the spectrograph has the ability to affect the final image in terms of sensitivity, reliability and calibration transferability (Qin *et al.,* 2013). The spectrograph passes light in the direction of the camera and detector. During the process it separates broadband light, which is necessary for detecting any changes in the light emitted (Reich, 2005). One aspect of the spectrograph that makes it exceptional

is the ability to provide spatial as well spectral information of the sample being analysed (Reich, 2005; Burger, 2006; Grahn & Geladi, 2007).

2.6.1.4 Camera

The camera configuration is an important component when using NIR hyperspectral imaging. To acquire the hyperspectral image, one of three camera configurations can be used specifically point scan, line scan and plane scan as illustrated in Figure 2.7.

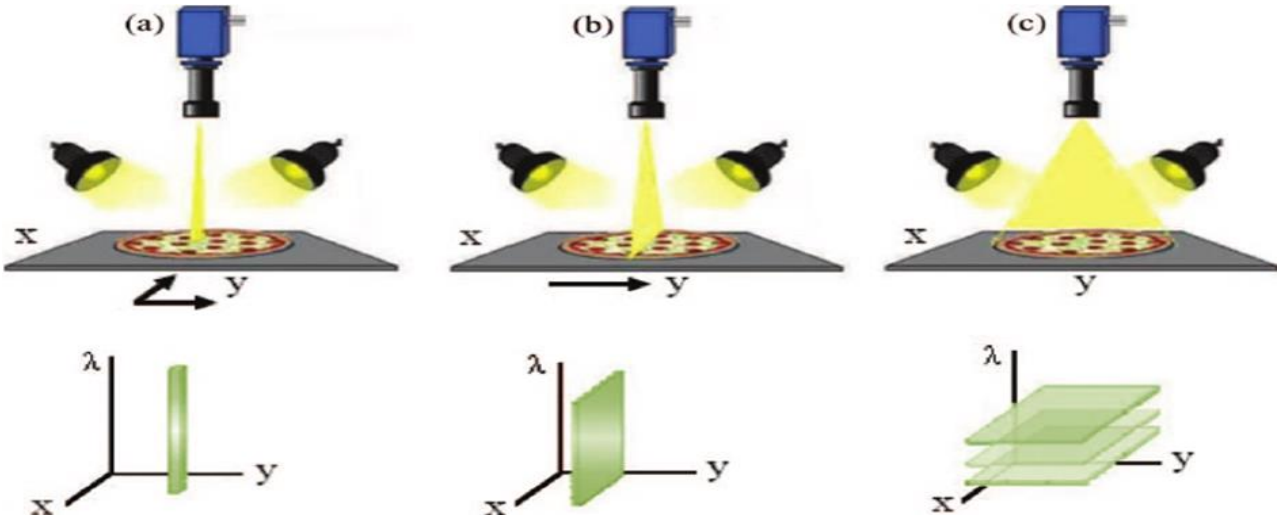


Figure 2.7 Three different camera configurations used during hyperspectral imaging. (**a**) Point scan, (**b**) line scan and (**c**) plane scan (adapted from ElMasry *et al.*, 2012).

The point scan set up, also known as the “whiskbroom”, is a camera configuration used to obtain a complete spectrum by imaging a sample one pixel at a time and the sample’s position has to be altered to recover the next spectrum (Gowen *et al.,* 2007). This is done automatically by the sample stage. This method can be time consuming, because scanning is done at each spatial position until the full specimen is imaged and leads to limited field view. Although it may take more time to use this technique, it yields comprehensive images with high spatial resolution (Geladi *et al.,* 2007; Gowen *et al.,* 2007). This technique is mostly used in microscopic imaging (Sahlin & Peppas, 1997).

The line scan or “pushbroom” configuration involves imaging an entire line of pixels by using a two-dimensional detector located above the sample horizontally to the sample stage (Burger, 2006; Gowen *et al.,* 2015). When light is reflected from the sample, the light passes through a slit leading in the direction of the detector. This configuration is mostly used in short wave infrared (SWIR) as well as visible-near infrared (Vis-NIR) hyperspectral imaging and is the more rapid method as no filter changes are needed. This method is fitting for online scanning on conveyor belts due to the two dimensional detector obtaining spectral and spatial data, line-by-line (Gowen *et al.*, 2008b).

The plane scan or “stare down” configuration involves a set up where the sample and spectrometer remain in a fixed position with the detector parallel to the surface of the sample (Gowen

et al., 2007). The sample is not moved during this configuration and the image field of view stays fixed. This configuration results in less background noise and generates a more reliable image (Burger, 2006). Imaging of the sample at each wavelength is recorded to obtain a hypercube, which consist of three-dimensional images stacked sequentially where one images represent a wavelength (Grahn & Geladi, 2007). For this configuration AOTFs and LCTFs are implemented, where AOTFs have fast scanning time and large spectral range, but LCTFs have exceptional image quality and a better spectral band pass (Gowen *et al.,* 2015). This scanning method was implemented on numerous occasions in the pharmaceutical industry as it offers several benefits (Roggo *et al.,* 2005).

The large data sets, which are generated using imaging spectroscopy can have drawbacks, mainly increasing the opportunity for an error to occur (Burger, 2006) and being complex to interpret. Fortunately, multivariate data analysis tools, such as chemometrics as well as multivariate data and image analysis can be used to investigate and reduce these data sets to be more user friendly.

2.7 Chemometrics, multivariate data and image analysis

Chemometrics is known as the science of extracting relevant chemical information from a vast amount of data obtained during spectroscopy by applying mathematical or statistical methods (Hand & Taylor, 1987). These techniques enable exploring the chemical information and by combining spectral information with chemical analysis or class information, models can be designed for predicting the chemical content or category of new samples (Massart *et al.,* 1988; Naes *et al.,* 2003; Wise *et al.,* 2006).

Another popular term used is multivariate data analysis (MDA) and is used interchangeably with chemometrics. When Chemometrics is applied to multivariate images, the process is known as multivariate image analysis (MIA). Analysis of images can be approached in two ways known as the pixel wise or object wise approach (Williams & Kucheryavskiy, 2016) and their application depends on the type of analysis and information required. The object wise approach uses the average spectra of all pixels and presents it as a data point. This approach is useful when the analysis of differences between samples is more interesting than the difference between pixels of a sample(s). The pixel wise approach uses the spectra from each pixel as a data point. This method is useful when chemical or physical differences within and between samples are of interest. Each method makes use of a different amount of data points to represent each sample (Kucheryavskiy, 2013; Bezuidenhout *et al.,* 2018).

Analysis of data can be divided into two categories and their use depends on the desired outcome. Unsupervised methods such as principal component analysis (PCA) are used to explore differences (chemical or physical) between samples. Supervised methods are used for specific outcomes such as classification of samples into categories or predicting the chemical contents of samples.

2.7.1 Unsupervised methods

2.7.1.1 PCA to explore differences

Principal component analysis (PCA) is the most popular, unsupervised technique used for data analysis and cluster visualization (Wold *et al.*, 1987; Kim H. Esbensen, Dominique Guyot, Frank Westad, 2002; Burger, 2006). PCA has the ability to reduce the dimensionality of a large data set and transform it to a smaller set of variables, which contain the most important information. During analysis, the hypercube obtained from hyperspectral imaging is first unfolded to create a new two-dimensional dataset (**X**) (Geladi *et al.,* 1989; Benito & Peña, 2005; Manley *et al.,* 2009). This is done by multiplying the spatial x and y (**I.J**), which is then plotted against the spectral λ variable (**K**) (Burger, 2006) as illustrated in Figure 2.8. The dataset can be normalized either by mean centering or using a scaling technique (e.g. autoscaling). Mean centering involves subtracting the mean of each variable from the values. This results in the mean of each variable being equal to zero and centers the data around the origin. Autoscaling uses mean-centering, followed by dividing each variable (or column) by the standard deviation of that column and is used for correcting different scaling methods and these methods assist to ensure that the main source of variance is the variable signal rather than noise (Wise *et al.*, 2006).

Next, the PCA scores and loadings are obtained, which is used to interpret the variance and can portray variability as well as the distributions of certain chemical or physical properties on images (Jolliffe, 1986; Bokobza, 1998; Gowen *et al.,* 2007). The scores and loadings, reconstructed by the data of the unfolded matrix X are obtained with Equation 2.1.

Equation 2.1:

$$X = TP' + E$$

where:

T = Score matrix

P' = Loadings matrix

E = Residual matrix

In Figure 2.8, **X** represents the dataset matrix with size **K** (wavelengths) x (I•J), **T** represents the score matrix, **P** the loading matrix and **E** the residual matrix containing the unwanted noise and other disturbances that can lead to a less accurate model (Manley *et al.,* 2009). The column vectors in P and in T can be easily interpreted through line and scatter plots. Scatter plots are used to identify similarities or differences between samples, while line plots are used to show relationships between variables and scores, which in turn give an indication of the most valuable variables (Naes *et al.,* 2003). Equation 2.1 can be illustrated geometrically as seen in Figure 2.8.

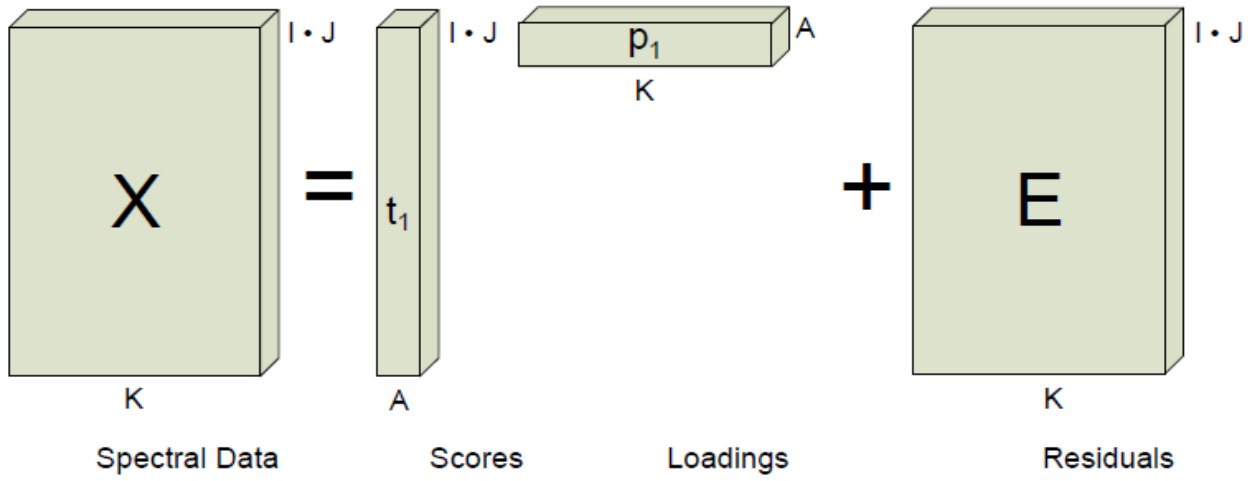


Figure 2.8 Geometrical illustration of a dataset matrix X with size (**K** x **(I • J)**), which can be reduced to the scores (**A** x (**I • J)**) and loadings **(K** x **A)** matrixes as well as the residuals (**K** x **(I • J)**) matrix. The reduced matrices contain all significant information about the data matrix **X** and are easier to interpret (adapted from Geladi, 2003; Du Toit, 2009).

PCA is a useful exploratory technique to illustrate differences in physical properties as well as chemical compounds within a sample. Principal component analysis has been successfully utilized in numerous studies. For example, PCA could be used to distinguish between different edible fats and oils (Dupuy *et al.,* 1996), identify organic pollutants in soil (Manz *et al.,* 2001), identify bruises on pickled cucumbers (Ariana *et al.,* 2006), monitor fermentation of red wine (Cozzolino *et al.,* 2006) and distinguish between different muscle types in various game animals (Dumalisile *et al.,* 2020). Principal component analysis has the advantage of reducing the number of variables and simplifying interpretation. However, interpretation of the loadings can sometimes be challenging, because several physical or chemical components can contribute to a single loading (Roggo *et al.,* 2005). Nonetheless, this technique is still widely used and very popular.

2.7.2 Supervised Methods

There are two types of supervised methods, namely regression and classification. The difference between the two is the type of output variables which are generated, where regression methods yield true values as an output (e.g. HU, protein, lipids, moisture) and classification methods divide variables based on a pre-established group or class (Wise *et al.,* 2006; Bezuidenhout *et al.,* 2018).

2.7.2.1 Partial least squares regression (PLSR)

Regression algorithms such as PLS can be used to yield values, such as HU as an output. All the wavelengths from the NIR spectrum is used by the PLSR method (full spectrum method) to predict sample composition (Wold *et al.,* 1987; Brereton, 2000). Regression algorithms can be applied to hyperspectral images to predict concentrations within a sample at a pixel level. This brings forth the ability to construct visual pixel maps (visual mapping method) used to show constituents'

quantity and distribution within a sample. Other regression methods include multiple linear regression (MLR) and principal component regression (PCR). The PLSR method is a good alternative to the more commonly used MLR and PCR, because it is more robust meaning that when new calibration samples are collected from the total sample population, the model parameters do not change that much (Geladi & Kowalski, 1986). Multiple linear regression pursues a single factor that correlates the predictor (X) variable with the predictor (Y) variable the best, for example the total amount of protein (Wise *et al.,* 2006).

The PLSR technique shows some similarities when compared to PCR and MLR. With PCR the objective is to find the highest amount of variance in predictor (X) within hyperplanes (Sun, 1995), while PLSR uses a linear regression model to estimate **B** (Equation 2.2) in a manner, which would increase the covariance between **X** and **Y** (Sjöström *et al.,* 1983). Otherwise stated, PLSR finds factors that captures variance (like PCR) and achieve correlation (like MLR) (Geladi & Kowalski, 1986; Burger, 2006). The PLSR technique can be illustrated by Equation 2.2. The regression equation can be used to build a regression model that can be used to predict the constituent concentration (Figure 2.9).

Equation 2.2:

$$\mathrm{Y} = \mathrm{XB} + \mathrm{E}$$

Y is the mean vector centered concentration, **X** is a matrix containing mean centered rows of spectra, **B** is the regression coefficients vector and **E** the residual matrix.

One of the most important aspects to keep in mind when using the PLSR method is the number of principal components (PC's) or latent variables. Selecting the number of PC's will have a direct effect on the covariance between X and Y. If the number of PC's are too small, the supplied signal will be reduced in the calibration model, which will lead to inadequate prediction. This phenomenon is known as underfitting. If the number of PC's are too high, excess signal including noise can be included in the calibration model leading to poor prediction as well. This occurrence is known as overfitting. Both of these circumstances might lead to build models, which work well for the calibration dataset, but when applied to the prediction of new samples the outcome will be inaccurate (Beebe *et al.,* 1998; Burger, 2006). Extracting meaningful information from the datasets require the use of preprocessing techniques to remove noise.

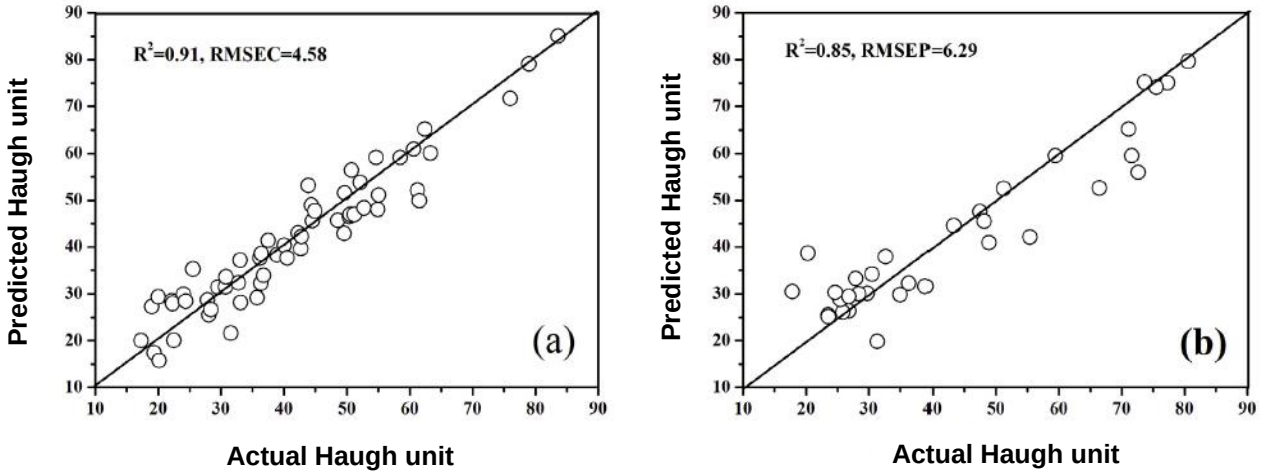


Figure 2.9 Illustration of the accuracy of a regression model when predicting the Haugh Unit (HU) of eggs versus the known HU content for eggs belonging to (**a**) the calibration set and (**b**) the prediction set (adapted from Suktanarak & Teerachaichayut, 2017). The closer the coefficient of determination (R^2) value is to 1 the more accurate the model. The error of the model is indicated by the Root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP).

2.7.3 Preprocessing techniques

Hyperspectral images contain important information about the sample, but also contain undesirable information, known as noise (Beebe *et al.,* 1998; Cen & He, 2007). Numerous sample properties can also contribute to unwanted variance captured in hyperspectral imaging spectra. This includes the sample size and shape, the consistency of the sample surface as well as the distance between the sample and the detector or camera (Burger, 2006; Heise & Winzen, 2007; Qin & Lu, 2008; Vidal & Amigo, 2012), unbalanced illumination or even due to instrumentation effects (Ozaki *et al.*, 2006; Nicolaï *et al.*, 2007; Heise & Winzen, 2007; Williams *et al.*, 2009).

The application of preprocessing techniques known as pretreatment methods can be used to transform spectral data and remove unwanted noise (Bro & Heimdal, 1996). Preprocessing result in increased signal-to-noise ratio in the data (Pizarro *et al.,* 2004). Various pretreatment categories exist and include baseline correction, noise reduction methods, linear transformation, scatter correction, variable centering, variable scaling, multivariate filtering and more (Barnes *et al.*, 1989; Martens & Stark, 1991; Guo *et al.*, 1999; Luypaert *et al.*, 2004; Ozaki *et al.*, 2006; Rinnan *et al.*, 2009; Fearn *et al.*, 2009). For the purpose of this thesis variable centering (mean-centering method), scatter correction (SNV and MSC method) and baseline correction (derivative method) will be discussed.

2.7.3.1 Mean centering

Mean centering presents one of the most commonly used methods to normalize or standardize data (Geladi, 2003). Mean centering involves centering data to a reference point and this is achieved by calculating the average of each variable and subtracting it from the data. In spectroscopy an average spectrum of the data set is calculated and subtracted from each spectrum. When implemented on each row of the data, that row will only display how it differs from the average data

of the initial data matrix (Wise *et al.,* 2006). Figure 2.10 shows how the raw data is transformed when mean-centering is applied using Equation 2.3. Where $\boldsymbol{X_c}$ is the centered data, **X** represents the data matrix, **1** the column vector and $\bar{\mathbf{x}}$ is the reference point or the average of the initial data set (Wise *et al.,* 2006). Mean-centering is usually applied, after other preprocessing to ensure noise is removed first before data centering.

Equation 2.3:

$$X_c = \mathrm{X} - 1\bar{\mathrm{x}}$$

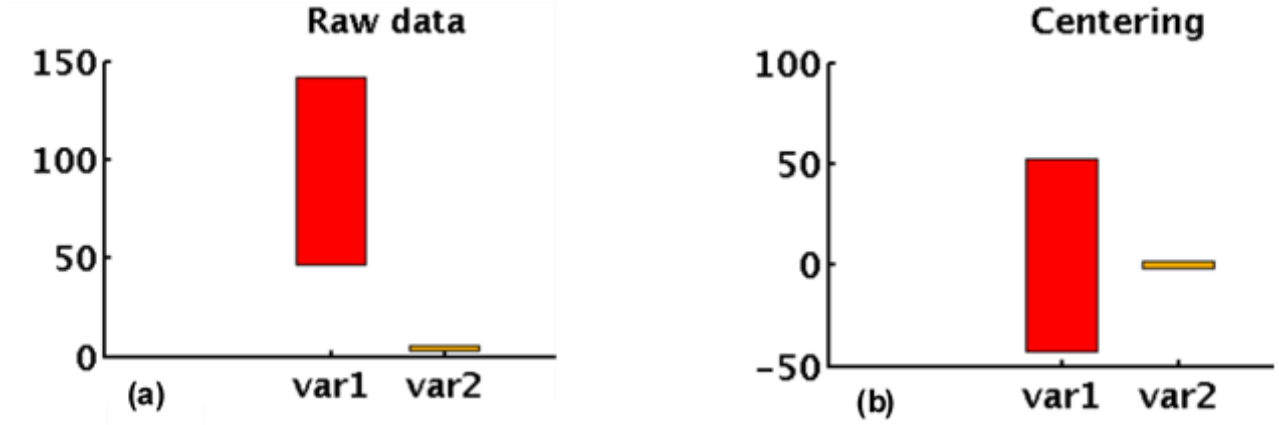


Figure 2.10 Illustration of the application of the mean-centering to the (**a**) original data matrix and (**b**) the resulting centered data.

2.7.3.2 Scatter correction preprocessing

Scatter correction removes light scattering, which occur due to differences in physical properties such as particle size and irregularities in sample surface or shape (Geladi *et al.,* 1985; Cen & He, 2007). This correction is known as multiplicative transformation and consists of various scatter correction methods, where the most popular techniques include standard normal variate (SNV) and multiplicative scatter correction (MSC) (Isaksson & Naes, 1988; Esquerre *et al.,* 2012). Standard normal variate is based on the fact that when the path length between samples in a data set vary, the spectra is normalized by SNV (Barnes *et al.,* 1989; Naes *et al.,* 2003). The SNV technique removes multiplicative effects (Rinnan *et al.*, 2009; Fearn *et al.*, 2009) by applying the algorithm displayed in (Equation 2.4).

Equation 2.4:

$$\mathrm{x}_{\mathrm{i,SNV}} = \left(\frac{\mathrm{x}_{\mathrm{ij}} - \bar{\mathrm{x}}_{\mathrm{i}}}{\mathrm{s}_{\mathrm{i}}}\right)$$

where $\mathbf{x_{ij}}$ is the initial spectrum, $\bar{\mathbf{x}}_{\mathbf{i}}$ is the mean spectrum, $\boldsymbol{s_i}$ is the standard deviation of each spectrum and $\mathbf{x_{i,SNV}}$ is the transformed spectrum.

Multiplicative scatter correction is established on the fact that scattering caused by physical light has different wavelength dependencies when compared to chemically absorbed light, thus MSC tries to separate these two (Geladi *et al.,* 1985; Isaksson & Naes, 1988; Williams *et al.,* 2009).

Multiplicative scatter correction was first introduced by generating a line of best fit from a single spectrum to a reference one. The spectra retrieved is adapted with the line of best fit's gradient (Martens & Næs, 1984). It is possible to categorize light responsible for absorption and scattering, with aid from data with a large number of wavelengths. Scattering from each individual sample is estimated relatively to an ideal sample so that all the samples are corrected to comparable scattering levels (Du Toit, 2009; Williams *et al.,* 2009). Martens and Naes (1984) showed that MSC uses linear regression between spectral variables and the average spectrum. All the spectra in the data set is used to determine the average spectra (Pizarro *et al.,* 2004; Rinnan *et al.,* 2009). Following this step, a least squares linear regression is carried out on the spectrum of the sample against the corresponding mean spectrum wavelength, resulting in a linear equation with a gradient and intercept (Equation 2.5). The value of the intercept is then subtracted from all the data points in the spectrum individually and subsequently dividing each individual absorbance value in the consequent spectrum by the gradient value (Equation 2.6) (Fearn *et al.,* 2009).

Equation 2.5:

$$x_{ij} = a_i + b_i \bar{x}_j + e_i$$

Equation 2.6:

$$x_{ij,MSC} = \left(\frac{x_{ij} - a_i}{b_i}\right)$$

where $\boldsymbol{x_{ij}}$ is the individual spectrum, $\boldsymbol{a_i}$ the intercept of least-squares regression, $\boldsymbol{b_i}$ the slope of least-squares regression, $\boldsymbol{\bar{x}_j}$ the mean spectrum and $\boldsymbol{e_i}$ is the residual spectrum. In Equation 2.6, $\boldsymbol{x_{ij,MSC}}$ is the transformed spectrum with a given number of wavelengths.

Multiplicative scatter correction pursues the removal of additive and multiplicative effects of the scatter in NIR spectra. This in turn leads to a reduction in spectral variations, which is not related to the differences in the concentration of the analytical compound (Pizarro *et al.,* 2004; Esquerre *et al.,* 2012).

2.7.3.3 Baseline corrections

One of the most popular baseline correction methods are derivatives. Derivatives are additive transformations used to increase the signal to noise (signal:noise) ratio. Noise, introduced by variation in lighting, instrumentation, sample size and more can be rectified by applying either first or second derivatives on the spectral data (Rinnan *et al.,* 2009; Wang & Zhou, 2011). The first and second derivates are implemented to correct for baseline shifts as well as separating peaks. The first derivative is responsible for removing the additive baseline, while the second derivative is responsible for the removal of the additive as well as the linear baseline variation in the initial data spectrum (Norris & Williams, 1984; Vidal & Amigo, 2012). The first and second derivative increases spectral resolution and removes noise by implementing Equation 2.7 and Equation 2.8 respectively. Savitzky-Golay is the most commonly used smoothing method (Savitzky & Golay, 1964; Shao &

Zhuang, 2004; Delwiche & Reeves, 2010; Zimmermann & Kohler, 2013). When the Savitzky-Golay prepossessing method is combined with other pretreatment techniques, it can produce higher spectral signals with less noise (Luypaert *et al.,* 2004; Wang *et al.,* 2006). When implementing these preprocessing techniques, it is important to select the correct polynomial order and window size. If the first derivative is used, the polynomial order will usually be two and the window size higher than the polynomial order (Cen & He, 2007; Bezuidenhout *et al.,* 2018). The spectral region which is used to define the smoothing preprocessing function is known as the window. Window size is of extreme importance, because selecting a too small window size can lead to excess unwanted noise and a too large window size can lead to exclusion of relevant sample information (Geladi *et al.,* 1999; Esquerre *et al.,* 2012).

Equation 2.7:

$$x'_i = x_i - x_{i-1}$$

Equation 2.8:

$$x''_i = x'_i - x'_{i-1} = x_{i-1} - 2\,(x_i) + x_{i+1}$$

where $\boldsymbol{x'_i}$ is the first derivative at a particular wavelength and $\boldsymbol{x''_i}$ is the second derivative at a particular wavelength (Cen & He, 2007). Preprocessing data is an important step towards developing more efficient models.

2.8 Application of multivariate image analysis for hyperspectral image analysis (HIA)

Analysis of hyperspectral images involve the application of several of the multivariate data analysis tools described in the previous section.

The following steps are implemented in the HIA process:

1. **Image correction.** Obtained images are adjusted by using white and dark references, followed by conversion of detector counts to pseudo absorbance values.
2. **Image cleaning/Selecting a region of interest.** Starting the HIA process, a default PCA model is created containing three PC's of the image. This results in development of score images, score plots and loading plots. The first PC describes the most variation (i.e. variation between eggs, dead pixels, shading, detector errors or background). Pixels associated with unwanted information can be removed using the interactive score images and score plots (Geladi *et al.,* 2004) to select the region of interest (ROI). As an example, the background pixels can be detected by selecting different regions or clusters within the score plot and the pixels associated with these data points would be displayed in the score image due to their interactive nature. Principal components can be plotted in different combinations against each other for the removal of unwanted pixels. Cleaning of the image,

leads to a new calculated PCA model with more visible patterns due to spreading of the remaining pixels in the score plot (Grahn & Geladi, 2007).

3. **Inclusion of additional PC's.** To search for more unwanted data, additional PC's can be added to the PCA model (normally no more than six PC's). Increased noise is usually described by higher order PC's, however it can also contain relevant information. For this reason, all possible PC combinations should be considered.

4. **Preprocessing techniques.** Preprocessing can be applied to the data, if necessary, for improved visualization. If the appropriate preprocessing technique is applied, it is possible to achieve better cluster identification.

5. **Examination of combinations of principal components in score plots.** Possible clustering of data can be identified by plotting different PCs against each other. Prior knowledge of the samples can assist with the identification of properties, which caused clustering. Pixels which overlap indicate that they have similar properties.

6. **Identification of clusters.** Labels can be assigned to clusters with the aid of prior knowledge of the image sample. By observing the interaction between the score plot and score image pixels associated with cluster(s) can be identified and assigned specific properties. This approach can be used to create 'maps of chemical or physical properties, which enable visualization of differences within or between samples.

7. **Classification of clusters in score plots.** Due to prior knowledge of the imaged sample, clusters that relate to differences or similarities can be identified and classified correspondingly. When the clusters are classified in the score plot, classification images are generated from score images. Classification images improves the visualization of chemical compound's distribution within a sample (Gowen *et al.*, 2008a; Williams *et al.*, 2009).

8. **Interpretation of loading line plots.** When a PCA model is constructed, loading line plots of each principal component is generated. The loading line plots along with score plots can assist with evaluating important absorption peaks, which may be useful to study clusters. The identity of functional groups associated with absorption peaks in the loadings line plot can be deduced by using absorption tables as illustrated by Osborne *et al.* (1993). This can assist with identification of compounds resulting in spectral variation within and between samples.

2.9 Conclusion

Quality and safety are the most important aspects in the egg industry. An ever-increasing demand for safe and high-quality foods is placed on the food industry in South Africa. The quality of eggs is dependent on various parameters, but the most important ones are the HU, protein, lipid and moisture composition. Hen age and storage duration can be the most influential elements affecting

these parameters and their effect should therefore be studied. It is therefore extremely important that quality assessment methods should be implemented that offers accuracy, rapid assessment, increased reliability and which is non-destructive. Conventional testing methods to determine egg quality can be expensive, time consuming, redundant and can lead to the destruction of the analysed samples. NIR hyperspectral imaging can potentially operate as an alternative screening method to differentiate between different egg qualities at an accelerated pace. The non-destructive characteristic of this method makes it possible to gain valuable information of samples without unnecessary wastage. The NIR hyperspectral imaging technique has the capability to conduct quantitative as well as qualitative analyses and show the distribution of compounds, which can be used to evaluate egg quality.

2.10 References

Abney, C., & Festing, L. C. 1881. On the influence of the atomic grouping in the molecules of organic bodies on their absorption in the infra-red region of the spectrum. Philos. Trans. R. Soc. London 172, 887–918.

Adesiyun, A., Offiah, N., Seepersadsingh, N., Rodrigo, S., Lashley, V., Musai, L., & Georges, K. 2005. Microbial health risk posed by table eggs in Trinidad. Epidemiol. Infect. 133, 1049–1056.

Ahn, D. U., Kim, S. M., & Shu, H. 1997. Effect of egg size and strain and age of hens on the solids content of chicken eggs. Poult. Sci. 76, 914–919.

Akbaş, Y., Altan, Ö., & Koçak, Ç. 1996. Effects of hen's age on external and internal egg quality characteristics. Turkish J. Vet. Anim. Sci. 20, 455–460.

Akter, Y., Kasim, A., Omar, H., & Sazili, A. Q. 2014. Effect of storage time and temperature on the quality characteristics of chicken eggs. J. Food, Agric. Environ. 12, 87–92.

Altan, Ö., Akbaş, Y., Sevgican, F., & Erkek, R. 1997. Effects of cold storage on egg quality. . Turkish J. Vet. Anim. Sci. 21, 335–339.

Amigo, J. M., Martí, I., & Gowen, A. 2013. Hyperspectral imaging and chemometrics. A Perfect combination for the analysis of food structure, composition and quality. Data handling in science and technology, 344–366.

Amigo, J. M., & Ravn, C. 2009. Direct quantification and distribution assessment of major and minor components in pharmaceutical tablets by NIR-chemical imaging. Eur. J. Pharm. Sci. 37, 76–82.

Anderson, W. T. 1951. Xenon compact are lamps. J. Opt. Soc. Am. 41, 385–388.

Ariana, D. P., Lu, R., & Guyer, D. E. 2006. Near-infrared hyperspectral reflectance imaging for detection of bruises on pickling cucumbers. Comput. Electron. Agric. 53, 60–70.

Balnave, D., Yoselewitz, I., & Dixon, R. J. 1989. Physiological changes associated with the production of defective egg-shells by hens receiving sodium chloride in the drinking water. Br. J. Nutr. 61, 35–43.

Barnes, R. J., Dhanoa, M. S., & Lister, S. J. 1989. Standard normal variate transformation and de-

trending of near-infrared diffuse reflectance spectra. Appl. Spectrosc. 43, 772–777.

Baum, W. A., & Dunkelman, L. 1950. Ultraviolet radiation of the high pressure xenon Arc*. J. Opt. Soc. Am. 40, 782–786.

Beardsworth, P. M., & Hernandez, J.-M. 2004. Yolk colour - An important egg quality attribute. Int. Poult. Prod. 12, 17–18.

Beebe, K. R., Pell, R. J., & Beth, M. 1998. Chemometrics: A practical guide by John Wiley & Sons: New York.

Bejaei, M., Wiseman, K., & Cheng, K. M. 2011. Influences of demographic characteristics, attitudes, and preferences of consumers on table egg consumption in British Columbia, Canada. Poult. Sci. 90, 1088–1095.

Benito, M., & Peña, D. 2005. A fast approach for dimensionality reduction with image data. Pattern Recognit. 38, 2400–2408.

Bergman, R. S., & Parham, T. G. 1993. Applications of thin film reflecting coating technology to tungsten filament lamps. IEE Proceedings, Part A Sci. Meas. Technol. 140, 418–428.

Bertechini, A. G., & Mazzuco, H. 2013. The table egg: a review. Ciência e Agrotecnologia 37, 115–122.

Bezuidenhout, C., Williams, P. J., & Rose, L. J. 2018. Differentiation of maize ear rot pathogens, on growth media, with near infrared hyperspectral imaging. Food Anal. Methods 12, 1556–1570.

Blanco, M., & Alcalá, M. 2006. Simultaneous quantitation of five active principles in a pharmaceutical preparation: Development and validation of a near infrared spectroscopic method. Eur. J. Pharm. Sci. 27, 280–286.

Blanco, M., & Villarroya, I. 2002. NIR spectroscopy: A rapid-response analytical tool. TrAC - Trends Anal. Chem. 21, 240–250.

Bokobza, L. 1998. Near infared spectroscopy. J. Near Infrared Spectrosc. 6, 3–17.

Boldrini, B., Kessler, W., Rebnera, K., & Kessler, R. W. 2012. Hyperspectral imaging: A review of best practice, performance and pitfalls for in-line and on-line applications. J. Near Infrared Spectrosc. 20, 483–508.

Bollengier-Lee, S., Mitchell, M. A., Utomo, D. B., Williams, P. E. V., & Whitehead, C. C. 1998. Influence of high dietary vitamin e supplementation on egg production and plasma characteristics in hens subjected to heat stress. Br. Poult. Sci. 39, 106–112.

Brabin, B. J., Hakimi, M., & Pelletier, D. 2001. Disorders of cholecalciferol metabolism in old egg-laying hens. J. Nutr. 131, 604-615.

Bradley, F. A., & King, A. J. 2016. Egg basics for the consumer: packaging, storage, and nutritional information.

Brake, J., Walsh, T., Benton, C., & Petitte, J. 1997. Egg handling and storage. Poult. Sci. 76, 144–151.

Brereton, R. G. 2000. Introduction to multivariate calibration in analytical chemistry. Analyst 125,

2125–2154.

Bro, R., & Heimdal, H. 1996. Enzymatic browning of vegetables. Calibration and analysis of variance by multiway methods. Chemom. Intell. Lab. Syst. 34, 85–102.

Bruce, J., & Drysdale, E. M. 1994. Trans-shell transmission. Microbiology of the Avian Egg.

Burger, J. E. 2006. Hyperspectral NIR image analysis, 63–91.

Burger, J., & Geladi, P. 2006. Hyperspectral NIR imaging for calibration and prediction: A comparison between image and spectrometer data for studying organic and biological samples. Analyst 131, 1152–1160.

Burley, R. W., & Vadehra, D. V. 1989. The avian egg : chemistry and biology. Wiley.

Catinhat, R. 2016. Egg anatomy and why it matters.

Cen, H., & He, Y. 2007. Theory and application of near infrared reflectance spectroscopy in determination of food quality. Trends Food Sci. Technol. 18, 72–83.

Charlton, B., Bermudez, A., Boulianne, M., Halvorson, D., Jeffrey, J., Newman, L., Sander, J., & Wakenell, P. 2000. Avian Disease Manual.

Chen, F., Wang, K., Qin, Z., Wu, D., Luo, X., & Liu, S. 2010. Design method of high-efficient LED headlamp lens. Opt. Express 18, 926–939.

Chen, S., Zhang, F., Ning, J., Liu, X., Zhang, Z., & Yang, S. 2015. Predicting the anthocyanin content of wine grapes by NIR hyperspectral imaging. Food Chem. 172, 788–793.

Cherian, G. 2008. Egg quality and yolk polyunsaturated fatty acid status in relation to broiler breeder hen age and dietary n-3 Oils. Poult. Sci. 87, 1131–1137.

Chien, Y. H., Chan, K. K., Yap, S. H. K., & Yong, K. T. 2018. NIR-responsive nanomaterials and their applications; upconversion nanoparticles and carbon dots: a perspective. J. Chem. Technol. Biotechnol. 93, 1519–1528.

Chung, S. H., & Lee, K. W. 2014. Effect of hen age, Storage duration and temperature on egg quality in laying hens. Int. J. Poult. Sci. 13, 634–636.

Coblentz, W. W. 1905. Infra-red absorption spectra, II. Phys. Rev. (Series I) 20, 337–363.

Cornforn, S. J., Parkison, T. L., & Robb, J. 1969. Rheological characteristics of processed whole egg. Int. J. Food Sci. Technol.

Cotteril, O. J., Chang, C. C., McBee, L. E., & Heymann, H. 1992. Metallic Cations affect functional performance of spray-dried heat-treated egg white. J. Food Sci. 57, 1321–1321.

Cozzolino, D., & Murray, I. 2004. Identification of animal meat muscles by visible and near infrared reflectance spectroscopy. LWT - Food Sci. Technol. 37, 447–452.

Cozzolino, D., Parker, M., Dambergs, R. G., Herderich, M., & Gishen, M. 2006. Chemometrics and visible-near infrared spectroscopic monitoring of red wine fermentation in a pilot scale. Biotechnol. Bioeng. 95, 1101–1107.

Curtis, P., Gardner, F., & Mellor, D. 1985. A Comparison of selected quality and compositional characteristics of brown and white shell eggs: I. shell quality. Poult. Sci., 297–301.

Curtis, P. A., Kerth, L. K., & Anderson, K. E. 2005. Quality and compositional characteristics of layer

hens as affected by bird age.

DAFF. 1999. Regulations regarding the grading, packing and marking of eggs destined for sale in the republic of South Africa.

Daniel, M., & Balnave, D. 1981. Responses of laying hens to gradual and abrupt increases in ambient temperature and humidity. Aust. J. Exp. Agric. 21, 189–195.

Dawes, C. M. 1975. Acid-base relationships within the avian egg. Biol. Rev. Camb. Philos. Soc. 50, 351–371.

Delwiche, S. R., & Reeves, J. B. 2010. A graphical method to evaluate spectral preprocessing in multivariate regression calibrations: example with savitzky-golay filters and partial least squares regression. Appl. Spectrosc. 64, 73–82.

Dumalisile, P., Manley, M., Hoffman, L., & Williams, P. J. 2020. Discriminating muscle type of selected game species using near infrared (NIR) spectroscopy. Food Control 110, 106981.

Dunn-Horrocks, S., Pichardo-Fuchs, M., Lee, J., Ruiz-Feria, C., Creger, C., Hyatt, D., Stringfellow, K., Sanchez, M., & Farnell, M. 2011. Effect of omega-3 enriched layer rations on egg quality. Int. J. Poult. Sci. 10, 8–11.

Dupuy, N., Duponchel, L., Huvenne, J. P., Sombret, B., & Legrand, P. 1996. Classification of edible fats and oils by principal component analysis of Fourier transform infrared spectra. Food Chem. 57, 245–251.

Dyrby, M., Engelsen, S. B., Nørgaard, L., Bruhn, M., & Lundsberg-Nielsen, L. 2002. Chemometric quantitation of the active substance (containing C≡N) in a pharmaceutical tablet using near-infrared (NIR) transmittance and NIR FT-Raman spectra. Appl. Spectrosc. 56, 579–585.

Ellis, J. W. 1929. Molecular absorption spectra of liquids below 3μ. Trans. Faraday Soc. 25, 888–898.

ElMasry, G., Kamruzzaman, M., Sun, D. W., & Allen, P. 2012. Principles and applications of hyperspectral imaging in quality evaluation of agro-food products: A Review. Crit. Rev. Food Sci. Nutr. 52, 999–1023.

ElMasry, G., & Sun, D. W. 2010. Principles of hyperspectral imaging technology. hyperspectral imaging for food quality analysis and control, 3–43.

Esquerre, C., Gowen, A. A., Burger, J., Downey, G., & O'Donnell, C. P. 2012. Suppressing sample morphology effects in near infrared spectral imaging using chemometric data pretreatments. Chemom. Intell. Lab. Syst. 117, 129–137.

Evans, R. J., Bandemer, S. L., & Davidson, J. A. 1966. Lipids and fatty acids in fresh and stored shell eggs. Biochem. Poult. Sci., 151–155.

Evans, R. J., Butts, H. A., Davidson, J. A., & Bandemer, S. L. 1949. The amino acid content of fresh and stored shell eggs. I. leucine, isoleucine, valine, glycine, serine, threonine, aspartic acid, and glutamic acid. Poult. Sci., 691–696.

Falconer, D. 1954. Validity of the theory of genetic correlation: an experimental test with mice. J. Hered. 45, 42–44.

Fearn, T., Riccioli, C., Garrido-Varo, A., & Guerrero-Ginel, J. E. 2009. On the geometry of SNV and MSC. Chemom. Intell. Lab. Syst. 96, 22–26.

Feng, Y. Z., Elmasry, G., Sun, D. W., Scannell, A. G. M., Walsh, D., & Morcy, N. 2013. Near-infrared hyperspectral imaging and partial least squares regression for rapid and reagentless determination of Enterobacteriaceae on chicken fillets. Food Chem. 138, 1829–1836.

Flethcer, D. L., Britton, W. M., Pesti, G. M., Rahn, A. P., & Savage, S. I. 1983. The relationship of layer flock age and egg weight on egg component yields and solids content. Poult. Sci. 62, 1800–1805.

Funk, E. M. 1944. Effects of temperature and humidity on the keeping quality of shell eggs. , 1–27.

Geladi, P. 2003. Chemometrics in spectroscopy. Part 1. Classical chemometrics. Spectrochim. Acta - Part B At. Spectrosc. 58, 767–782.

Geladi, P., Bärring, H., Dåbakk, E., Trygg, J., Antti, H., Wold, S., & Karlberg, B. 1999. Calibration transfer for predicting lake-water pH from near infrared spectra of lake sediments. J. Near Infrared Spectrosc. 7, 251–264.

Geladi, P., Burger, J., & Lestander, T. 2004. Hyperspectral imaging: Calibration problems and solutions. Chemometrics and intelligent laboratory systems, 209–217.

Geladi, P. L. ., Grahn, H. F., & Burger, J. E. 2007. Multivariate images, hyperspectral images: background and equipment. Techniques and applications of hyperspectral image analysis. Techniques and Applications of Hyperspectral Image. 18th ed, 590–598.

Geladi, P., Isaksson, H., Lindqvist, L., Wold, S., & Esbensen, K. 1989. Principal component analysis of multivariate images. Chemom. Intell. Lab. Syst. 5, 209–220.

Geladi, P., & Kowalski, B. R. 1986. Partial least-squares regression: A tutorial. Elsevier Science Publishers B.V.

Geladi, P., MacDougall, D., & Martens, H. 1985. Linearization and scatter-correction for near-infrared reflectance spectra of meat. Appl. Spectrosc. 39, 491–500.

Gennadios, A., Handa, A., Froning, G. W., Weller, C. L., & Hanna, M. A. 1998. Physical properties of egg white-dialdehyde starch films †. J. Agric. Food Chem 46, 1297–1302.

Goetz, A. F. H., Vane, G., Solomon, J. E., & Rock, B. N. 1985. Imaging spectrometry for earth remote sensing. Science (80-.). 228, 1147–1153.

Gowen, A. A., Feng, Y., Gaston, E., & Valdramidis, V. 2015. Recent applications of hyperspectral imaging in microbiology. Talanta 137, 43–54.

Gowen, A. A., O'Donnell, C. P., Cullen, P. J., & Bell, S. E. J. 2008a. Recent applications of Chemical Imaging to pharmaceutical process monitoring and quality control. Eur. J. Pharm. Biopharm. 69, 1–51.

Gowen, A. A., O'Donnell, C. P., Cullen, P. J., Downey, G., & Frias, J. M. 2007. Hyperspectral imaging - An emerging process analytical tool for food quality and safety control. Trends Food Sci. Technol. 18, 590–598.

Gowen, A. A., O'Donnell, C. P., Taghizadeh, M., Cullen, P. J., Frias, J. M., & Downey, G. 2008b. Hyperspectral imaging combined with principal component analysis for bruise damage detection on white mushrooms [Agaricus bisporus). In Journal of Chemometrics, 259–267.

Grahn, H. F., & Geladi, P. 2007. Techniques and applications of hyperspectral image analysis.

Grashorn, M. 2016. Effects of storage conditions on egg quality. 50, 23–25.

Guo, Q., Wu, W., & Massart, D. L. 1999. The robust normal variate transform for pattern recognition with near-infrared data. Anal. Chim. Acta 382, 87–103.

Hall. 1944. Egg shell color in crosses between white-and brown-egg breeds. Poult. Sci. 23, 259–265.

Hammershøj, M., & Qvist, K. B. 2001. Importance of hen age and egg storage time for egg albumen foaming. LWT - Food Sci. Technol. 34, 118–120.

Hand, D. J., & Taylor, C. C. 1987. Multivariate analysis of variance and repeated measures.

Hart, J. R., & Norris, K. H. 1961. Determination of the moisture content of seeds by near-infrared spectrophotometry of their methanol extracts. Cereal Chem. 39, 93–94.

Hart, J. R., Norris, K. H., & Golumbic, G. 1962. Determination of the moisture content of seeds by near-infrared spectrophotometry of their methanol extracts. Cereal Chem. 39, 94–99.

Haugh, R. . 1937. The Haugh unit of measuring egg quality. US Poult. Mag 43, 522–555, 572–573.

Heiman, V., & Carver, J. S. 1936. The Albumen Index as a physical measurement of observed egg quality. Poult. Sci., 141–148.

Heise, H. M., & Winzen, R. 2007. Chemometrics in near-infrared spectroscopy. Near-Infrared Spectroscopy. Wiley-VCH Verlag GmbH, Weinheim, Germany, 125–162.

Henderson, W. 1941. Studies in egg quality. II. Determinations of albumen index, yolk index and yolk colour, thickness of egg shell and egg shape.

Herschel, W. 1800. Investigation of the powers of the prismatic colours to heat and illuminate objects; with remarks, that prove the different refrangibility of radiant heat. to which is added, an inquiry into the method of viewing the sun advantageously, with telescopes of large apertures and high magnifying powers. Philos. Trans. R. Soc. London, 90, 255–283.

Hill, A. T., & Hall, J. W. 1980. Effects of various combinations of oil spraying, washing, sanitizing, storage time, strain, and age of layer upon albumen quality changes in storage and minimum sample sizes required for their measurement. Poult. Sci. 59, 2237–2242.

Hocking, P. M., Bain, M., Channing, C. E., Fleming, R., & Wilson, S. 2003. Genetic variation for egg production, egg quality and bone strength in selected and traditional breeds of laying fowl. Br. Poult. Sci. 44, 365–373.

Holman, R. T., Johnson, S. B., & Hatch, T. F. 1982. A case of human linolenic acid deficiency involving neurological abnormalities. Am. J. Clin. Nutr. 144–147.

Hunter, R. 1958. Photoelectric color difference meter*. J. Opt. Soc. Am. 48, 985.

Ibarz, A., Sangronis, E., Barbosa-Cánovas, G. V., & Swanson, B. G. 1999. Textura de geles de huevo obtenidos por alta presión / Texture of egg gels induced by high hydrostatic pressure.

Food Sci. Technol. Int.

Isaksson, T., & Naes, T. 1988. Effect of multiplicative scatter correction (MSC) and linearity improvement in NIR spectroscopy. Appl. Spectrosc. 42, 1273–1284.

Jacob, J. P., Wilson, H. R., Miles, R. D., Butcher, G. D., & Mather, F. B. 2003. Factors affecting egg production in backyard chicken flocks. EDIS Publ. Syst., 1–8.

Jin, Y. H., Lee, K. T., Lee, W. I., & Han, Y. K. 2011. Effects of storage temperature and time on the quality of eggs from laying hens at peak production. Asian-Australasian J. Anim. Sci. 24, 279–284.

Jolliffe, I. T. 1986. Principal components in regression analysis, 129–155.

Kamel, B., Bond, C., & Diab, M. 1980. Egg quality as affected by storage and handling methods. J. Food Qual. 3, 261–273.

Kaminska, B. Z., & Skraba, B. 1991. Analysis of hen types considering albumen: Yolk ratio and its change during the laying cycle. Natl. Res. Inst. Anim. Prod. Dep. Nutr. Physiol. Sarego 2, 43–45.

Kamruzzaman, M., ElMasry, G., Sun, D. W., & Allen, P. 2013. Non-destructive assessment of instrumental and sensory tenderness of lamb meat using NIR hyperspectral imaging. Food Chem. 141, 389–396.

Karoui, R., Kemps, B., Bamelis, F., De Ketelaere, B., Decuypere, E., & De Baerdemaeker, J. 2006. Methods to evaluate egg freshness in research and industry: A review. Eur. Food Res. Technol. 222, 727–732.

Kim H. Esbensen, Dominique Guyot, Frank Westad, L. P. H. 2002. Multivariate data analysis : in practice : an introduction to multivariate ... 6th editio.

Kim, M. S., Lefcourt, A. M., & Chen, Y.-R. 2003. Multispectral laser-induced fluorescence imaging system for large biological samples. Appl. Opt. 42, 3927–3934.

Kim, M. H., Rushmeier, H., Dorsey, J., Harvey, T. A., Prum, R. O., Kittle, D. S., & Brady, D. J. 2012. 3D imaging spectroscopy for measuring hyperspectral patterns on solid objects. ACM Trans. Graph. 31, 1–11.

Kirunda, D. F. K., & McKee, S. R. 2000. Relating quality characteristics of aged eggs and fresh eggs to vitelline membrane strength as determined by a texture analyzer. Poult. Sci. 79, 1189–1193.

Knox, C. W., & Godfrey, A. B. 1937. Factors influencing the percentage of thick albumen of hens' eggs. Poult. Sci., 159–162.

Kochav, S., & Eyal-Giladi, H. 1971. Bilateral symmetry in chick embryo determination by gravity 1027-1029. Science (80-.). 171, 1027–1029.

Koehler, F. W., Lee, E., kidder, L. H., & Lewis, E. N. 2002. Near infrared spectroscopy: the practical chemical imaging solotion. Spectrosc. Eur. 14, 12–19.

Kucheryavskiy, S. 2013. A new approach for discrimination of objects on hyperspectral images. Chemom. Intell. Lab. Syst. 120, 126–135.

Kul, S., & Seker, I. 2004. Phenotypic correlations between some external and internal egg quality

traits in the Japanese quail (Coturnix coturnix japonica). Int. J. Poult. Sci.

Kumar, S., & Mittal, G. S. 2010. Rapid detection of microorganisms using image processing parameters and neural network. Food Bioprocess Technol. 3, 741–751.

Ledur, M. C., Liljedahl, L. E., McMillan, I., Asselstine, L., & Fairfull, R. W. 2002. Genetic effects of aging on egg quality traits in the first laying cycle of White Leghorn strains and strain crosses. Poult. Sci. 81, 1439–1447.

Leeson, S., Summers, J. D., & Caston, L. 1993. Response of brown-egg strain layers to dietary calcium or phosphorus. Poult. Sci. 72, 1510–1514.

Linden, G., Lorient, D., Bichat, H., & Carballo García, F. J. 1996. Agroindustrial biochemistry: food revaluation of agricultural production. Acribia, Zaragoza.

Liu, S., Zhang, X., & Sun, S. 2005. Discrimination and feature selection of geographic origins of traditional Chinese medicine herbs with NIR spectroscopy. Chinese Sci. Bull. 50, 179–184.

Luypaert, J., Heuerding, S., Heyden, Y. Vander, & Massart, D. L. 2004. The effect of preprocessing methods in reducing interfering variability from near-infrared measurements of creams. J. Pharm. Biomed. Anal. 36, 495–503.

Lyon, R. C., Lester, D. S., Lewis, E. N., Lee, E., Yu, L. X., Jefferson, E. H., & Hussain, A. S. 2002. Near-infrared spectral imaging for quality assurance of pharmaceutical products: Analysis of tablets to assess powder blend homogeneity. AAPS PharmSciTech 3, 1–15.

Manley, M., Williams, P., Nilsson, D., & Geladi, P. 2009. Near infrared hyperspectral imaging for the evaluation of endosperm texture in whole yellow maize (Zea maize L.) Kernels. J. Agric. Food Chem. 57, 8761–8769.

Mann, K., & Mann, M. 2008. The chicken egg yolk plasma and granule proteomes. Proteomics 8, 178–191.

Manz, M., Wenzel, K. D., Dietze, U., & Schüürmann, G. 2001. Persistent organic pollutants in agricultural soils of central Germany. Sci. Total Environ. 277, 187–198.

Martens, H., & Næs, T. 1984. Multivariate calibration. I. Concepts and distinctions. Trends Anal. Chem. 3, 204–210.

Martens, H., & Stark, E. 1991. Extended multiplicative signal correction and spectral interference subtraction: New preprocessing methods for near infrared spectroscopy. J. Pharm. Biomed. Anal. 9, 625–635.

Martin, M. E., Wabuyele, M. B., Chen, K., Kasili, P., Panjehpour, M., Phan, M., Overholt, B., Cunningham, G., Wilson, D., Denovo, R. C., & Vo-Dinh, T. 2006. Development of an advanced Hyperspectral Imaging (HSI) system with applications for cancer detection. Ann. Biomed. Eng. 34, 1061–1068.

Martinsen, P., & Schaare, P. 1998. Measuring soluble solids distribution in kiwifruit using near-infrared imaging spectroscopy. Postharvest Biol. Technol. 14, 271–281.

Massart, D. L., Vandeginste, B. G. M., Deming, S. N., Michotte, Y. &, & Kaufman, L. 1988. Chemometrics: A Textbook. Amsterdam: Elsevier Science Publishers.

McGoverin, C. M., Engelbrecht, P., Geladi, P., & Manley, M. 2011. Characterisation of non-viable whole barley, wheat and sorghum grains using near-infrared hyperspectral data and chemometrics. Anal. Bioanal. Chem. 401, 2283–2289.

McNally, E. H. 1943. The origin and structure of the vitelline membrane of the domestic fowl's egg. Poult. Sci. 22, 40–43.

McNaughton, S. A., & Marks, G. C. 2002. Selenium content of Australian foods: A review of literature values. J. Food Compos. Anal. 15, 169–182.

Mehl, P. M., Chen, Y. R., Kim, M. S., & Chan, D. E. 2004. Development of hyperspectral imaging technique for the detection of apple surface defects and contaminations. J. Food Eng. 61, 67–81.

Menesatti, P., Antonucci, F., Pallottino, F., Roccuzzo, G., Allegra, M., Stagno, F., & Intrigliolo, F. 2010. Estimation of plant nutritional status by Vis-NIR spectrophotometric analysis on orange leaves [Citrus sinensis (L) Osbeck cv Tarocco]. Biosyst. Eng. 105, 448–454.

Menezes, J. C., Ferreira, A. P., Rodrigues, L. O., Brás, L. P., & Alves, T. P. 2009. Chemometrics role within the pat context: examples from primary pharmaceutical manufacturing. comprehensive chemometrics, 313–355.

Miller, C. E. 2001. Chemical principles of near infrared technology (K Norris & PC Williams, Eds.). In: Near I. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA, Minnesota, USA.

Mine, Y. 1995. Recent advances in the understanding of egg white protein functionality. Trends Food Sci. Technol. 6, 225–232.

Monira, K. N., Salahuddin, M., & Miah, G. 2003. Effect of breed and holding period on egg quality characteristics of chicken. Int. J. Poult. Sci. 2, 261–263.

Moran, T., & Hale, H. P. 1935. Physics of the hen's egg: I. Membranes in the egg. Poult. Sci., 35–42.

Morris, T. R. 1985. The manipulation of egg size and egg quality. S. Afr. J. Anim. Sci. 15, 120-122.

Moussa, M., Martinet, V., Trimeche, A., Tainturier, D., & Anton, M. 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: Cryoprotective effect on frozen-thawed bull semen. Theriogenology 57, 1695–1706.

Muir, F. V., Gerry, R. W., & Harris, P. C. 1975. Effect of various sources and sizes of calcium carbonate on egg quality and laying house performance of red × rock sex-linked females. Poult. Sci. 54, 1898–1904.

Naes, T., Isakson, T., Fearn, T., & Davies, T. 2003. A user-friendly guide to multivariate calibration and classification. J. Chemom., 114–125.

Nicolaï, B. M., Beullens, K., Bobelyn, E., Peirs, A., Saeys, W., Theron, K. I., & Lammertyn, J. 2007. Nondestructive measurement of fruit and vegetable quality by means of NIR spectroscopy: A review. Postharvest Biol. Technol. 46, 99–118.

Norris, K., & Williams, P. 1984. Optimization of mathematical treatments of raw near-infrared signal

in the measurement of protein in hard red spring wheat. I. Influence of particle size. Cereal Chem. 61, 158–165.

O'Malley, B. 2005. Clinical Anatomy and Physiology of Exotic Species.

Omana, D. A., Liang, Y., Kav, N. N. V., & Wu, J. 2011. Proteomic analysis of egg white proteins during storage. Proteomics 11, 144–153.

OnbaŞılar, E. E., Erdem, E., Poyraz, O., & Yalcin, S. 2011. Effects of hen production cycle and egg weight on egg quality and composition, hatchability, duckling quality, and first-week body weight in Pekin ducks. Poult. Sci. 90, 2642–2647.

Ortiz-Somovilla, V., España-España, F., Gaitán-Jurado, A. J., Pérez-Aparicio, J., & De Pedro-Sanz, E. J. 2007. Proximate analysis of homogenized and minced mass of pork sausages by NIRS. Food Chem. 101, 1031–1040.

Osborne, B. G., Fearn, T., & Hindle, P. H. 1993. Practical NIR spectroscopy with applications in food and beverage analysis. Harlow, England: Longman Scientific & Technical.

Owens, B., McCann, M. E. E., McCracken, K. J., & Park, R. S. 2009. Prediction of wheat chemical and physical characteristics and nutritive value by near-infrared reflectance spectroscopy. Br. Poult. Sci. 50, 103–122.

Owren, P. A., Hellem, A. J., & Ödegaard, A. 1964. Linolenic acid for the prevention of thrombosis and myocardial infraction. Lancet, 975–979.

Ozaki, Y., Mcclure, W. F., & Christy, A. A. 2006. Near-infrared spectroscopy in food science and technology.

Pan, L., Tu, K., Zhao, L., Zhao, Y., & Pan, X. 2005. Preliminary research of chicken egg crack detection based on acoustic resonance analysis. Nongye Gongcheng Xuebao/Transactions Chinese Soc. Agric. Eng.

Pasquini, C. 2003. Near infrared spectroscopy: Fundamentals, practical aspects and analytical applications. J. Braz. Chem. Soc. 14, 198–219.

Patterson, J. T. 1909. An experimental study on the development of the vascular area of the chick blastoderm. 16, 83–90.

Pizarro, C., Esteban-Díez, I., Nistal, A. J., & González-Sáiz, J. M. 2004. Influence of data preprocessing on the quantitative determination of the ash content and lipids in roasted coffee by near infrared spectroscopy. Anal. Chim. Acta 509, 217–227.

Poggenpoel, D. G., Ferreira, G. F., Hayes, J. P., & Du Preez, J. J. 1996. Response to long-term selection for egg production in laying hens. Br. Poult. Sci. 37, 743–756.

Powrie, W. D., & Nakai, S. 1986. The chemistry of eggs and egg products. Egg Science and technology, 97–139.

Qin, J. 2010. Hyperspectral imaging instruments. Hyperspectral imaging for food quality analysis and control, 129–172.

Qin, J., Chao, K., Kim, M. S., Lu, R., & Burks, T. F. 2013. Hyperspectral and multispectral imaging for evaluating food safety and quality. J. Food Eng. 118, 157–171.

Qin, J., & Lu, R. 2008. Measurement of the optical properties of fruits and vegetables using spatially resolved hyperspectral diffuse reflectance imaging technique. Postharvest Biol. Technol. 49, 355–365.

Reich, G. 2005. Near-infrared spectroscopy and imaging: Basic principles and pharmaceutical applications. Adv. Drug Deliv. Rev. 57, 1109–1143.

Reis, L. H., Gama, L. T., & Chaveiro Soares, M. 1997. Effects of short storage conditions and broiler breeder age on hatchability, hatching time, and chick weights. Poult. Sci. 76, 1459–1466.

De Reu, K., Grijspeerdt, K., Messens, W., Heyndrickx, M., Uyttendaele, M., Debevere, J., & Herman, L. 2006. Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including Salmonella enteritidis. Int. J. Food Microbiol. 112, 253–260.

Rinnan, A., Van Den Berg, F., & Engelsen, S. B. 2009. Review of the most common preprocessing techniques for near-infrared spectra. Trends Anal. Chem. 28, 1201–1222.

Robert, J. R. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. J. Poult. Sci. 41, 161–177.

Roggo, Y., Edmond, A., Chalus, P., & Ulmschneider, M. 2005. Infrared hyperspectral imaging for qualitative analysis of pharmaceutical solid forms. Anal. Chim. Acta 535, 79–87.

Roland, D. A. 1979. Factors influencing shell quality of aging hens. Poult. Sci.

Romanoff, A. L., & Romanoff, A. J. 1949. The avian egg.

Rose-Martel, M., Du, J., & Hincke, M. T. 2012. Proteomic analysis provides new insight into the chicken eggshell cuticle. J. Proteomics 75, 2697–2706.

Sahlin, J. J., & Peppas, N. A. 1997. Near-field FTIR imaging: A technique for enhancing spatial resolution in FTIR microscopy. J. Appl. Polym. Sci. 63, 103–110.

Sakudo, A., Kuratsune, H., Kobayashi, T., Tajima, S., Watanabe, Y., & Ikuta, K. 2006. Spectroscopic diagnosis of chronic fatigue syndrome by visible and near-infrared spectroscopy in serum samples. Biochem. Biophys. Res. Commun. 345, 1513–1516.

Samli, H. E., Agma, A., & Senkoylu, N. 2005. Effects of storage time and temperature on egg quality in old laying hens. J. Appl. Poult. Res. 14, 548–553.

SAPA. 2017. Key market signals in the egg industry for the fourth quarter of 2017. , 1–29.

Savitzky, A., & Golay, M. J. E. 1964. Smoothing and differentiation of data by simplified least squares procedures. Anal. Chem. 36, 1627–1639.

Schoor, A. van, Pieterse, E., & Hoffman, L. C. 2017. The assessment of black soldier fly (*Hermetia illucens*) pre-pupae , grown on human faecal waste , as a protein source in broiler and layer diets. , 106.

Scott, H. M., & Warren, D. C. 1941. The relation of total weight and the weight of the component parts of the egg to hatching power. Poult. Sci., 75–78.

Scotter, C. N. G. 1997. Non-destructive spectroscopic techniques for the measurement of food quality. Trends Food Sci. Technol. 8, 285–292.

Selle, P. H., & Ravindran, V. 2007. Microbial phytase in poultry nutrition. Anim. Feed Sci. Technol. 135, 1–41.

Serranti, S., Gargiulo, A., & Bonifazi, G. 2012. Classification of polyolefins from building and construction waste using NIR hyperspectral imaging system. Resour. Conserv. Recycl. 61, 52–58.

Shahin, M. A., & Symons, S. J. 2008. Detection of hard vitreous and starchy kernels in Amber durum wheat samples using hyperspectral imaging (GRL number M306). NIR news 19, 16–18.

Shao, X., Bian, X., Liu, J., Zhang, M., & Cai, W. 2010. Multivariate calibration methods in near infrared spectroscopic analysis. Anal. Methods 2, 1662–1666.

Shao, X., & Zhuang, Y. 2004. Determination of chlorogenic acid in plant samples by using near-infrared spectrum with wavelet transform preprocessing. Anal. Sci. 20, 451–454.

Sharp, P. F., & Powell, C. K. 1930. Decrease in interior quality of hens' eggs during storage as indicated by the yolk. Ind. Eng. Chem. 22, 908–910.

Shibata, S., Ohdan, H., Noriyuki, T., Yoshioka, S., Asahara, T., & Dohi, K. 1999. Novel assessment of acute lung injury by in vivo near-infrared spectroscopy. Am. J. Respir. Crit. Care Med. 160, 317–323.

Sierra, V., Aldai, N., Castro, P., Osoro, K., Coto-Montes, A., & Oliván, M. 2008. Prediction of the fatty acid composition of beef by near infrared transmittance spectroscopy. Meat Sci. 78, 248–255.

Silversides, F. G., & Scott, T. A. 2001. Effect of storage and layer age on quality of eggs from two lines of hens. Poult. Sci. 80, 1240–1245.

Sim, J. S. 1998. Designer eggs and their nutritional and functional significance. In: Simopoulos, A.P. (Ed.), The return of omega-3 fatty acids into the food supply, 1. land-based animal food products and their health effects. , 89–101.

Sisaka, C., Csanádia, Z., Rónay, E., & Szajáni, B. 2006. Elimination of glucose in egg white using immobilized glucose oxidase. Enzyme Microb. Technol. 39, 1002–1007.

Siska, J., & Hurburgh, C. R. 1995. Corn density measurement by near-infrared transmittance. Trans. Am. Soc. Agric. Eng. 38, 1821–1824.

Sjöström, M., Wold, S., Lindberg, W., Persson, J. Å., & Martens, H. 1983. A multivariate calibration problem in analytical chemistry solved by partial least-squares models in latent variables. Anal. Chim. Acta 150, 61–70.

Sobamiwa, O. 1998. Performance and egg quality of hens fed cocoa husk based diet . Niger. J. Anim. Prod. 25, 22–24.

Song, W. O., & Kerver, J. M. 2000. Nutritional contribution of eggs to american diets. J. Am. Coll. Nutr. 29, 556–562.

Spahn, G., Plettenberg, H., Nagel, H., Kahl, E., Klinger, H. M., Mückley, T., Günther, M., Hofmann, G. O., & Mollenhauer, J. A. 2008. Evaluation of cartilage defects with near-infrared

spectroscopy (NIR): An ex vivo study. Med. Eng. Phys. 30, 285–292.

Sparks, N. H. C. 2005. The hen's egg-is its role in human nutrition changing?

Stadelman, W. J. 1986. The preservation of quality in shell eggs. Egg science and technology. CRC Press, 63–73.

Stadelman, W. J., & Cotterill, O. J. 1977. Egg science and technology.

Suktanarak, S., & Teerachaichayut, S. 2017. Non-destructive quality assessment of hens' eggs using hyperspectral images. J. Food Eng. 215, 97–103.

Sun, J. 1995. A correlation principal component regression analysis of NIR data. J. Chemom. 9, 21–29.

Surai, P. F., McDevitt, R. M., Speake, B. K., & Sparks, N. H. C. 1999. Carotenoid distribution in tissues of the laying hen depending on their dietary supplementation. WProc. Nutr. Soc. 5830A.

Tankeu, S., Vermaak, I., Chen, W., Sandasi, M., & Viljoen, A. 2016. Differentiation between two 'fang ji' herbal medicines, Stephania tetrandra and the nephrotoxic Aristolochia fangchi, using hyperspectral imaging. Phytochemistry 122, 213–222.

Tatzer, P., Wolf, M., & Panner, T. 2005. Industrial application for inline material sorting using hyperspectral imaging in the NIR range. Real-time imaging 11, 99–107.

Tazawa, H., & Whittow, C. 2000. Incubation physiology. Sturkie's avian physiology, 617–632.

Tilki, M., & Saatci, M. 2004. Effects of storage time on external and internal characteristics in partridge (*Alectoris graeca*) eggs. Rev. Med. Vet. (Toulouse). 155, 561–564.

Du Toit, G. 2009. Near infrared hyperspectral imaging and chemometrics for exploration and classification of whole wheat kernels. , 110.

Toussant, M. J., & Latshaw, J. D. 1999. Ovomucin content and composition in chicken eggs with different interior quality. J. Sci. Food Agric. 79, 1666–1670.

Travel, A., Nys, Y., & Bain, M. 2011. Effect of hen age, moult, laying environment and egg storage on egg quality.in Improving the safety and quality of eggs and egg products: Egg chemistry, production and consumption.

Valous, N. A., Mendoza, F., Sun, D. W., & Allen, P. 2009. Colour calibration of a laboratory computer vision system for quality evaluation of pre-sliced hams. Meat Sci. 81, 132–141.

Vargas, C. R. S., Ruiz, S. L. C., & Navas, L. M. C. 2018. Merging Manual and Automated Egg Candling: A Safety and Social Solution. Enfoque UTE 9, 70–76.

Vermeulen, P., Fernández Pierna, J. A., van Egmond, H. P., Dardenne, P., & Baeten, V. 2012. Online detection and quantification of ergot bodies in cereals using near infrared hyperspectral imaging. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 29, 232–240.

Vidal, M., & Amigo, J. M. 2012. Preprocessing of hyperspectral images. Essential steps before image analysis. Chemom. Intell. Lab. Syst. 117, 138–148.

Vogliano, C., & Brown, K. 2016. The State of America's wasted food and opportunities to make a difference. J. For. -washingt., 0–28.

Vuilleumier, J. P. 1969. The ‘roche yolk colour fan‘—An instrument for measuring yolk colour. Poult. Sci. 48, 767–779.

Walsh, K. B., & Kawano, S. 2009. Near-infrared spectroscopy. In: optical monitoring of fresh and processed agricultural crops (M Zude, Ed.). New York: CRC Press.

Wang, L., Lee, F. S. C., Wang, X., & He, Y. 2006. Feasibility study of quantifying and discriminating soybean oil adulteration in camellia oils by attenuated total reflectance MIR and fiber optic diffuse reflectance NIR. Food Chem. 95, 529–536.

Wang, W., & Paliwal, J. 2007. Near-infrared spectroscopy and imaging in food quality and safety. Sens. Instrum. Food Qual. Saf. 1, 193–207.

Wang, Y., Wei, L., Yuan, L., Li, C., Lv, G., Xie, F., Han, G., Shu, R., & Wang, J. 2016. New generation VNIR/SWIR/TIR airborne imaging spectrometer. Hyperspectral remote sens. Appl. Environ. Monit. Saf. Test. Technol.

Wang, X., & Zhou, G. 2011. Study on pretreatment algorithm of near infrared spectroscopy. IFIP Advances in Information and Communication Technology, 623–632.

Washburn, K. W. 1982. Incidence, cause, and prevention of egg shell breakage in commercial production. Poult. Sci. 61, 1982.

Weichman, F. L., Schoot, J., & Kenway, D. J. 1997. Egg candling system.

Williams, P., Antoniszyn, J., & Manley, M. 2019. Near infrared technology : getting the best out of light.

Williams, P., Geladi, P., Fox, G., & Manley, M. 2009. Maize kernel hardness classification by near infrared (NIR) hyperspectral imaging and multivariate data analysis. Anal. Chim. Acta 653, 121–130.

Williams, P. J., & Kucheryavskiy, S. 2016. Classification of maize kernels using NIR hyperspectral imaging. Food Chem. 209, 131–138.

Wise, B. M., Gallagher, N. B., Bro, R., Shaver, J. M., Windig, W., & Koch, R. S. 2006. Chemometrics tutorial for PLS toolbox and solo.

Wold, S., Esbensen, K., & Geladi, P. 1987. Principal component analysis.

Wolfenson, D., Frei, Y. F., Snapir, N., & Berman, A. 1981. Heat stress effects on capillary blood flow and its redistribution in the laying hen. Pflügers Arch. Eur. J. Physiol. 390, 86–93.

Woodcock, T., Downey, G., & O’Donnell, C. P. 2008. Better quality food and beverages: The role of near infrared spectroscopy. J. Near Infrared Spectrosc. 16, 1–29.

Woolley, J. T. 1971. Reflectance and transmittance of light by leaves. Plant physiol. 47, 656–662.

Workman, J. 1993. A brief review of the near infrared measurement technique. NIR news 4, 8–16.

Wu, D., & Sun, D. W. 2013. Advanced applications of hyperspectral imaging technology for food quality and safety analysis and assessment: A review - Part I: Fundamentals. Innov. Food Sci. Emerg. Technol. 19, 1–14.

Xiao, J. F., Zhang, Y. N., Wu, S. G., Zhang, H. J., Yue, H. Y., & Qi, G. H. 2014. Manganese supplementation enhances the synthesis of glycosaminoglycan in eggshell membrane: A strategy to improve eggshell quality in laying hens. Poult. Sci. 93, 380–388.

Xin, H., Gates, R. S., Puma, M. C., & Ahn, D. U. 2002. Drinking water temperature effects on laying hens subjected to warm cyclic environments. Poult. Sci.

Zimmermann, B., & Kohler, A. 2013. Optimizing savitzky-golay parameters for improving spectral resolution and quantification in infrared spectroscopy. Appl. Spectrosc. 67, 892–902.

Chapter 3
The effect of hen age on egg quality parameters

Abstract

This study investigated the effect of hen age on certain internal and external egg quality parameters. For this trial, 80 Amberlink hen eggs were collected from eight different age groups (eight treatments with 10 eggs per treatment) which included ages 21, 29, 37, 45, 53, 61, 69 and 77 weeks. Eggs were subjected to physical quality analysis to assess internal and external egg quality as well as the proximate composition. Data collected for external quality investigation included egg weight, height, diameter, shell thickness, shell weight and shell bumps. Results showed that hen age had a significant effect on all the external egg quality parameters. Data collected to determine internal egg quality included yolk height, thick albumen height, thin albumen height, yolk weight, albumen weight, Haugh unit, thick albumen spread, thin albumen spread, yolk colour L*, yolk colour a*, yolk colour b*, Roche yolk colour fan score, vitelline membrane integrity, blood spots and meat spots. Hen age had a significant effect on all the internal egg quality parameters except for thick albumen spread, yolk colour b*, vitelline membrane integrity, blood spots and meat spots. The most pronounced differences in egg quality were observed in eggs laid between 21 and 29 weeks of age for most of the parameters, which can be ascribed to hens reaching maturity. Hen age also significantly influenced the proximate parameters which included the moisture, protein, lipid content but had no effect on the ash content. It can be concluded that hen age had a significant effect on most of the internal, external and proximate quality parameters proving that hen age is an important factor to consider when assessing egg quality.

Keywords: *albumen quality, Haugh unit, Roche colour fan, vitelline membrane, yolk colour, yolk quality*

3.1 Introduction

Eggs are an inexpensive food source, which are rich in vitamins, minerals and most importantly protein (Cook & Briggs, 1986; Patil *et al.*, 2005). Eggs are also known for supplying all essential amino acids (Layman & Rodriguez, 2009), is a reliable source of fatty acids and contains low cholesterol levels (Stadelman, 1992), making it ideal for human consumption. Egg quality depends on both the internal and external egg quality parameters. There are numerous factors that can affect egg quality characteristics. These factors can include the strain of the bird (Silversides & Scott, 2001; Singh *et al.*, 2009), anatomy (Ocak *et al.*, 2004; Incharoen *et al.*, 2009), endocrinology (Bacon & Skala, 1968; Dickerman & Bahr, 1989), diseases (Messens *et al.*, 2005; Messens *et al.*, 2007), nutrition (Quentin *et al.*, 2004; Al-Shami *et al.*, 2012), mycotoxins (Abdelhamid & Dorra, 1990; Rizzi *et al.*, 2003), lighting program or lighting source (Leeson *et al.*, 1982; Kamanli *et al.*, 2015),

temperature (Williams, 1992; Akter *et al.*, 2014), humidity (Daniel & Balnave, 1981; Yahav *et al.*, 2000), management practices (Mench *et al.*, 1986; Abrahamsson & Tauson, 1995) to only name a few. These factors are known to have an effect on certain egg quality parameters but hen age's effect has proven to have a greater influence in numerous studies executed over the years (Rossi & Pompei, 1995; Silversides & Scott, 2001; Bar *et al.*, 2002; Robert, 2004; Akyurek & Okur, 2009; Bozkurt & Tekerli, 2009; Travel *et al.*, 2011).

Within commercial genotypes, hen age is one of the main factors that can influence the weight as well as the size of egg (Williams, 1992; Chung & Lee, 2014). As hens age, the portion (%) of yolk increases and the portion (%) of albumen decreases in the egg resulting in a lower albumen to yolk ratio (Ternes *et al.*, 1944; Suk & Park, 2001). In terms of egg shell, older hens produce thinner shells with shorter pores when compared with younger hens (Britton, 1977). It was suggested by Robert (2004) that older hens lay thinner shelled eggs compared to younger ones, because the amount of shell deposited or calcification (the process of depositing calcium carbonate) stays the same between ages. Egg weight, along with egg height and diameter, thus increase with no proportional increase in calcification, resulting in thinner shells as egg size increase (Ternes *et al.*, 1944; Rodriguez-Navarro *et al.*, 2002). For this reason, eggs laid by older hens are often downgraded due to higher incidences of broken or cracked shells.

Internal egg quality is divided into yolk quality and albumen quality. There are two main factors used to describe yolk quality i.e. vitelline membrane strength and yolk colour. The vitelline membrane surrounds and protects the yolk, which is why the strength of the vitelline membrane is important because it gives an indication of how easily the yolk would break. Ruptured egg yolks is a criteria, which customers use to judge the quality of store bought eggs on (Beardsworth *et al*., 2004; Roberts, 2005). Colour is another criterion with importance to the consumer. Yolk colour can be determined with a Roche yolk colour fan or a digital colour spectrophotometer. There are many controversies surrounding the colour of egg yolks. Yolk colour preference is usually linked to geographical locations, but most consumers prefer yolks of a darker colour (Beardsworth & Hernandez, 2004). A survey done in Canada revealed that most consumers prefer darker yolk colours, as it is perceived to be heathier (Bejaei *et al.*, 2011), while a study done in Ethiopia revealed that most consumers preferred lighter (yellow) yolk colours, also believing it is the healthier choice (Senbeta *et al.*, 2015). No research could be found that suggested that darker yolks are heathier than yellow yolks and *vice versa*, but studies have shown that pasture raised hens laid eggs with darker yolks containing more omega-3 fatty acids and vitamins (Castellini *et al.*, 2002; Dvořák *et al.*, 2010). The level of carotenoids is the main factor that influences yolk colour. Carotenoids can be included in the diets of layers by using certain raw materials. This means that the yolk colour can directly be influenced by the diet, depending on the carotenoid content in their feed (Silversides & Scott, 2001; Beardsworth & Hernandez, 2004). The level of carotenoids absorbed by the small intestine of hens and incorporated in the yolk is believed to be influenced by the age of the hen as well (Cherian, 2008). The presence of blood spots and meat spots can also be used as an indication of egg quality. Blood

spots are caused by haemorrhages during ovulation, which result in blood clots that are visible as a distinct red speckle or speckles that varies in size and quantity depending on the intensity of the haemorrhage (Nalbandov & Card, 1943). Burmester and Card (1937) found that meat spots are formed when blood clots are transformed due to changes in pH and high temperatures after ovulation, during the egg formation process, which was also confirmed by Nalbandov and Card (1943). However, no resent research could be found on the formation of meat spots but Stadelman (1986) suggested that meat spots are blood spots that lost their colour. Blood spots and meats spots can be found attached to the yolk surface, whilst in the albumen it is located between the thick and thin albumen layers (Jeffrey, 1945; Grashorn, 2016).

The albumen quality is mainly determined by the thick albumen height and albumen viscosity. The albumen quality can be expressed using the Haugh unit (HU) scale, which can be calculated by using a logarithm of the thick albumen height, adjusted to the egg weight (Haugh, 1937). Changes in the albumen height can be attributed to changes in the lysozyme-ovomucin complex, which is influenced by an unknown mechanism in eggs laid by older hens (Kato *et al.*, 1970; Hayakawa *et al.*, 1983; Toussant & Latshaw, 1999). There are some nutritional factors that can affect the albumen quality, but nutrition does not have a substantial effect on general internal egg quality (Williams, 1992; Youssef *et al.*, 2013; Saki *et al.*, 2014).

External egg quality is related to the quality of the shell and is usually assessed through visualization (Tůmová *et al.*, 2014). Egg shell quality is any characteristic (cracked shells, pigmentation, etc.) of the shell that would influence consumer acceptability (Stadelman & Cotterill, 1977; Silversides & Scott, 2001; Bar *et al.*, 2002; Robert, 2004). The quality of the shell can be assessed in several ways, however some of the techniques are destructive. Factors affecting egg shell quality include egg size or egg weight, which are directly related to hen age (Roland, 1979), storage time, genotype and moult, but the most important factor is nutrition (Roberts, 2004). Calcium carbonate ($CaCO_3$) and phosphorus (P) is necessary for shell formation to prevent cracks and deformation (Rabon *et al*., 1990), while vitamin D is required for the metabolism of residual calcium from the bones (Bar & Hurwitz *et al*., 1987).

Hen age has an effect not only on the internal and external egg quality parameters, but also on the proximate constituents. It is well established that a large egg consist out of 31% yolk, 58% albumen and 11% shell, and on average an egg contains roughly 75% moisture (Stadelman & Cotterill, 1977). The protein and lipid content is affected by the yolk to albumen ratio, which depends on the egg size (Washburn, 1979; Silversides & Scott, 2001). Hen age can thus affect the protein and lipid content, since egg weight increases with hen age (Flethcer *et al.*, 1983). Ahn *et al.* (1997) stated that hen age can also influence the moisture content, due to a change in the amount of solid (dry matter) to moisture content ratio.

Since hen age can have a substantial impact on the quality, it is important to assess to what extent certain egg quality parameters are influenced by this factor. Although numerous studies have been done to evaluate the impact of hen age on egg quality parameters such as egg weight, HU and

albumen height, little research has been done on hen age's effect on other quality characteristics such as yolk colour, albumen spreading distance, proximate composition, to only name a few. The research aim for this chapter was therefore to determine the effect of hen age on various internal and external egg quality parameters. The results obtained in this research trial will also provide insight into egg quality. Furthermore, the reference values obtained in the current research trial will be used in a subsequent analysis, using near infrared (NIR) hyperspectral imaging with the aid of principal component analysis and partial least squares regression to quantify and distinguish egg qualities, as discussed in Chapter 5.

3.2 Materials and Methods

3.2.1 Experimental design

For the purpose of this trial 480 Amberlink hen eggs were collected from Rosendal Poultry Farm situated 34 km from Stellenbosch University near Paarl (-33.738734, 19.029038). Information such as diet composition, feed intake, average daily gain, housing conditions and lighting program was not disclosed by the farm due to security purposes and will therefore not be discussed in the current study. However, hens received the same diet irrespective of age. All the eggs were collected on the day they were laid. Eight groups of 60 eggs per group were collected from the farm and transported to Stellenbosch University, where the eggs were subjected to NIR hyperspectral imaging at the Department of Food Sciences (-33.925242, 18.871121), discussed in Chapter 5. This was followed by egg quality measurements at the Department of Animal Sciences (-33.931567, 18.867191). No cracked eggs were collected. The only difference between the eight egg groups (eight treatments) were the age of the hen. The youngest group of hens were 21 weeks of age and the oldest 77 weeks of age, with each consecutive age group being eight weeks older than the previous (21, 29, 37, 45, 53, 61 69 and 77 weeks of age). Each treatment (age group) consisted of 60 eggs of which ten were used for immediate analysis, resulting in a final sample size of 80 eggs. The other eggs were stored to evaluate the effect of storage time on egg quality characteristics (Chapter 4).

3.2.2 Experimental procedure and data collection

On the day of collection each egg was marked with a soft pencil with a number, date and age of the hen. All the eggs were subjected to internal- and external egg quality parameters (Table 3.1). Eggs were weighed using a laboratory scale (Mettler PC 4400 scale) and placed in a temperature (15.5˚C ± 2.2˚C) and humidity (75.8% ± 3.2%) storage room at the Mike de Vries building (-33.931567, 18.867191).

The external quality assessment commenced by weighing all the eggs followed by size classification, according to South African standards as explained in Chapter 2 (Table 2.1). The presence of bumps and bands were recorded for each egg. Categorical data (Table 3.1) was not

quantitatively measured but recorded as present or not present except for the Roche yolk colour fan which consisted of a colour scale ranging from 1 to 16. The egg height and diameter were measured in millimetres (mm) using a digital calliper (Mitutoyo 500-196-20, 150 mm, 0.02 accuracy).

After external evaluation, the egg was broken out of its shell on a level flat glass surface. Underneath the glass was a white surface with six concentric circles as shown in (Figure 3.1). These circles were used to determine the thick and thin albumen spreading distance from the egg yolk centre. Four measurements were taken at the furthest point at the top, left, bottom and right-hand side for both thick and thin albumen spread. These four measurements were averaged and assigned to an albumen spread category (Table 3.2).

Table 3.1 Egg quality parameters recorded

External Quality Parameters		Internal Quality Parameters	
Whole egg	Egg shell	Egg yolk	Egg albumen
[a]Weight (g)	[a]Weight (g)	[a]Weight (g)	[a]Weight (g)
[a]Height (mm)	[a]Thickness (mm)	[a]Height (mm)	[a]Thick Albumen Height (mm)
[a]Diameter (mm)	[b]Bumps	[b]Roche Colour Fan	[a]Thin Albumen Height (mm)
	[b]Banded	[a]Colour L*	[a]Thick Albumen Spread (mm)
		[a]Colour a*	[a]Thin Albumen Spread (mm)
		[a]Colour b*	[b]Meat Spots
		[b]Vitelline Membrane	[b]Blood Spots
		[b]Meat Spots	
		[b]Blood Spots	
		[b]Mottled	
		[b]Double Yolk	

[a]Numerical data; [b]Categorical data; *Colour L = lightness/darkness; a = red/green; b = yellow/blue

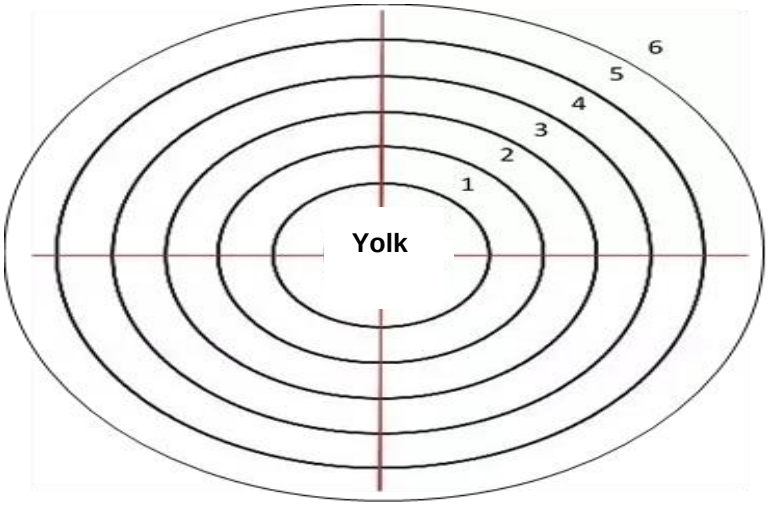


Figure 3.1 Graphic illustration of the thick and thin albumen spread grid used to categorize thick and thin albumen spreading distance.

Table 3.2 Categories used to evaluate the thick and thin albumen spreading properties

Albumen Spread Category:	Distance in Millimetre (mm)
1	$25 \leq \bar{x} > 75$
2	$75 \leq \bar{x} > 165$
3	$165 \leq \bar{x} > 185$
4	$185 \leq \bar{x} > 205$
5	$205 \leq \bar{x} > 225$
6	$225 \leq \bar{x} > 245$

If the yolk ruptured when the egg was broken out of the shell, it indicated that the vitelline membrane integrity was compromised and was recorded as either intact or not intact. The yolk was checked for meat spots, blood spots, mottling and double yolks. The albumen was checked for blood spots. If any of these incidences were present, it was recorded.

Empty shell weight was recorded using a laboratory scale (Mettler PC 4400 balance). Shell thickness was determined by recording three measurements along the edge of the shell using a digital calliper. The three measurements were averaged to obtain the final shell thickness measurement.

Yolk height, thick albumen height and thin albumen height was measured in millimetres (mm) using a manual tripod micrometer. After the yolk height was recorded the yolk was carefully removed using vacuum suction ensuring that it remains intact and was transferred to a petri dish and the weight recorded. Albumen weight was calculated by subtracting the shell and yolk weight from the total egg weight (Equation 3.1).

The yolk colour was determined using a digital colour spectrophotometer and a Roche colour fan (Vuilleumier, 1969). The digital colour spectrophotometer was used in accordance with the Hunter scale, shown in Figure 3.2 (Hunter,1958). Before spectrophotometer measurements took place, the spectrophotometer was calibrated as specified by the manufactures (HunterLab, 2020). For digital colour measurements the petri dish was placed onto a black surface. The lens of the digital colour spectrophotometer was placed as closely as possible to the surface of the yolk when taking a reading, making sure it does not touch the surface. This is done to ensure that the lens is not damaged or tainted and allows accurate consecutive readings to be recorded. The Hunter colour scale is in a cube form, where L* is only limited to a numeric scale in the cube ranging from 0 to 100. The L* axis at the bottom is black (0) and at the top white (100). The closer the value is to 100 the lighter the sample is and *vice versa*. The a* and b* axes are not limited to a specific range. A negative a* value indicates greenness and a positive a* value indicates redness. A negative b* value indicates blueness and a positive b* value indicates yellowness. The colour fan consists of 16 yellow colour variation schemes ranging from light yellow to dark yellow, also known as the Roche yolk colour scale (Vuilleumier, 1969). The yolk colour fan however is a combination between the three digital colour measurements L*, a* and b* (Vuilleumier, 1969). The colour fan gives an overall view of the yolk colour since it is difficult for the user's eyes to distinguish between different L* a* and b* colours.

When the yolk colour fan was used, it was placed as closely as possible to the yolk and a visual score is recorded.

After colour determination, the calculation of the HU followed. This HU can be used to determine egg freshness and can be calculated by using Equation 3.2. The HU ranges from 0 to 130. Once the HU is obtained a Haugh grade can be assigned to the egg, where HU above 72 is an 'AA'–grade, between 72 and 60 an 'A'-grade and below 60 a 'B'-grade (USDA, 2000). Haugh Unit can also be used as an indication if the egg is fresh or not. According to Zhao *et al*. (2010) eggs are classified as "fresh" when the HU is above 60 and below 60 is classified as "unfresh".

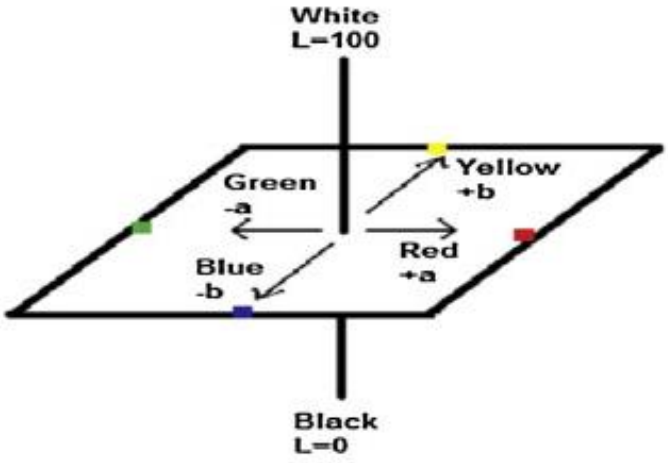


Figure 3.2 Illustration of the Hunter colour scale containing the L, a and b dimensions (Abd-Elhady, 2014).

Equation 3.1:

Albumen weight (g) = Whole egg weight (g) - Yolk weight (g) – Shell weight (g)

Equation 3.2:

$$HU = 100.\log(h-1.7w^{0.37}+7.6)$$

where:

h = The height of the thick albumen

w = Total egg weight

After all the internal and external parameters were recorded, the egg yolk and albumen of the same sample were homogenised and subjected to proximate analysis (Section 3.2.3).

3.2.3 Proximate analysis

All the chemical analysis took place at the Department of Animal Science, Stellenbosch University (-33.931567, 18.867191). All gravimetric measurements (Equation 3.3 to 3.7) were done in grams (g). The dry matter (DM), ash, crude fat (acid hydrolysis) and crude protein (CP) determination methods was performed as stipulated by the Association of Official Analytical Chemists (AOAC, 2002).

Eggs were freeze dried prior to proximate analysis. The homogenised internal egg contents (yolk and albumen) of each sample was transferred to different plastic containers, covered with plastic film containing pin sized holes to facilitate moisture extraction. Samples were freeze dried for 48 hr to ensure all the moisture was removed. The freeze dryer method was selected for moisture extraction, because of the high moisture content in eggs (Moscona, 1950), but mainly to prevent protein structure damage, which can occur in oven drying (Meehan *et al*., 1961). The total moisture of each egg was calculated with Equation 3.3. After freeze drying, the solid (moisture free) sample was homogenised and subjected to DM, ash, lipid and protein analysis.

The DM (official method 934.01) content of the samples was obtained by using Equation 3.4. From each sample, 2 g was weighed in duplicate and placed in a pre-weighed, moisture free porcelain crucible. Thereafter, the crucibles containing the sample was placed in a 100˚C oven for 24 hr and transferred to a desiccator for 30 min to cool down before being weighed.

Equation 3.3:

$$\%\text{Moisture} = \frac{((\text{Empty crucible weight (g)} + \text{Moisture free sample weight (g)}) - \text{Crucible and dry sample weight (g)})}{\text{Moisture free sample weight (g)}} \times 100$$

Equation 3.4:

$$\%\text{DM} = 100 - \%\text{Moisture}$$

The ash content of the egg samples was determined using Equation 3.5 (Official method 942.05). Samples were placed in a combustion oven for 6 hr at 500˚C, left to cool for 2 hr, placed in a desiccator for 30 min and then weighed.

Equation 3.5:

$$\%\text{Ash} = \frac{\text{Crucible and ash weight (g)} - \text{Empty and moisture free crucible weight (g)}}{\text{Sample weight (g)}} \times 100$$

$$\%\text{Organic matter} = 100 - \%\text{Ash}$$

Crude fat determination was done using the acid hydrolysis official method 925.32 (AOAC, 2002). Two gram per sample was weighed in duplicate, transferred to test tubes and 2 mL ethanol (99%) and 10 mL hydrogen chloride (HCl) (25 mL, 38% HCl & 11 mL dist. H_2O) added to each sample. The test tubes were kept in a water bath for 30 min, left to cool for 30 min and then placed in a separation funnel. The test tubes were rinsed with 10 mL ethanol (99%) and added to the funnel. Thereafter, 25 mL of diethyl ether was added and the tubes were shaken by hand for 1 min and 25 mL of petroleum ether was added after, shaking once more for 1 min. The funnels were left for 1 min for the liquids to separate and the top portion was poured into a pre-weighed, moisture free fat beaker. This step was repeated twice, using only 15 mL of diethyl ether and 15 mL of petroleum ether. After the last extraction, the fat beakers were placed on a sand bath to evaporate all the ether. Beakers were then placed in a desiccator for half an hour and then weighed. Equation 3.6 was used to calculate the crude fat.

Equation 3.6:

$$\%\text{Lipid} = \frac{\text{Fat cup and fat weight (g) - Empty and moisture free fat cup weight (g)}}{\text{Sample weight (g)}} \times 100\%$$

Crude protein (CP) was determined by measuring the total amount of nitrogen (N) in a sample using the LECO FP528 apparatus (Official method 4.207). All samples were analysed in duplicate, where 1.5 g of a sample was weighed in a foil cup, placed into the LECO FP528 and the total nitrogen content was read directly from the machine. A conversion factor of 6.25 was used to calculate the protein content for eggs as suggested by Jones (1931). Crude protein was then calculated using Equation 3.7.

Equation 3.7:

$$\%\text{CP} = \text{Nitrogen}(\%) \times 6.25$$

3.2.4 Statistical analysis

For statistical analysis the data analysis software, STATISTICA, Version 9 (TIBICO Software Inc., 2018) was used. A one-way analysis of variances (ANOVA) was used to analyse the main effect (hen age) on egg quality parameters. The Bonferroni least significant difference (LSD) *post hoc* test was used to see if there were any differences between the means of different ages in terms of the variables. A probability level of 5% ($P \leq 0.05$) was accepted as being statistically significant. The incidence of mottled yolks, double yolks and banded eggshells were too low to allow for statistical analysis. When internal egg contents were investigated it was noted that all blood spot incidences occurred on both the yolk and albumen. The same was observed for meat spots. For this reason, yolk and albumen blood spots were combined and subjected to statistical analysis. The same was done for yolk and albumen meat spots. Figure 3.3 to Figure 3.7 were used for the sole purpose to show more visible differences between hen ages, as the results depicted in Table 3.3 to Table 3.5 indicates the absolute values for the various egg quality parameters for each hen age group.

3.3 Results and Discussion

3.3.1 Effect of hen age on external egg quality parameters

The results depicted in Table 3.3 showed that the age of the hen significantly influenced ($P \leq 0.05$) all the external parameters, which include total egg weight, egg height, egg diameter, shell thickness, shell weight and shell bumps. The presence of bumps was recorded as categorical data and is therefore not displayed in Table 3.3.

The whole egg weight (weight of shell and contents) in this study ranged from 45 g to 64 g between the eight age groups and was significantly influenced ($P<0.01$) by the age of the hen. As stated by Chung and Lee (2014), egg weight increases with an increase in hen age resulting in a rise of heterogeneity, which is clearly visible in Figure 3.3. The results in Table 3.3 indicate the absolute values of egg weight between the different ages hens and for this reason Figure 3.3 was

Table 3.3 The means ± standard deviations of external egg quality parameters for eggs laid by Amberlink hens with ages ranging from 21 to 77 weeks

Parameter		Egg Weight (g)	Egg Height (mm)	Egg Diameter (mm)	Shell thickness (mm)	Shell Weight (g)
Treatment (Hen Age in Weeks)	**21**	$45.02^{e} \pm 1.70$	$49.71^{d} \pm 1.40$	$40.95^{b} \pm 1.34$	$0.49^{a} \pm 0.04$	$6.62^{d} \pm 0.42$
	29	$59.41^{d} \pm 1.63$	$53.84^{c} \pm 1.52$	$44.00^{a} \pm 1.21$	$0.44^{bc} \pm 0.04$	$8.05^{c} \pm 0.52$
	37	$61.70^{b} \pm 1.91$	$55.40^{ab} \pm 1.31$	$44.25^{a} \pm 1.32$	$0.45^{b} \pm 0.03$	$8.10^{c} \pm 0.34$
	45	$60.01^{cd} \pm 1.85$	$55.05^{b} \pm 1.25$	$44.58^{a} \pm 1.28$	$0.44^{bc} \pm 0.03$	$8.12^{c} \pm 0.49$
	53	$60.68^{bcd} \pm 1.66$	$55.33^{ab} \pm 1.13$	$44.43^{a} \pm 0.96$	$0.42^{bcd} \pm 0.03$	$8.00^{c} \pm 0.43$
	61	$61.01^{bc} \pm 1.76$	$55.97^{ab} \pm 0.96$	$44.60^{a} \pm 0.89$	$0.40^{d} \pm 0.04$	$8.22^{bc} \pm 0.31$
	69	$63.80^{a} \pm 1.50$	$55.94^{ab} \pm 1.53$	$44.94^{a} \pm 1.23$	$0.42^{cd} \pm 0.02$	$8.58^{ab} \pm 0.52$
	77	$64.12^{a} \pm 1.01$	$56.35^{a} \pm 1.39$	$44.71^{a} \pm 1.07$	$0.39^{d} \pm 0.02$	$8.68^{a} \pm 0.60$
	P value	<0.01	<0.01	<0.01	<0.01	<0.01

($^{a, b, c, d, e}$) Means with different superscripts within the same column differ significantly (P≤0.05).

included to illustrate more clear differences between the hen ages in terms of whole egg weight. The biggest increase in size is between the age of 21 and 29 weeks. These results are in agreement with previous studies (Hill & Hall, 1980; Silversides & Scott, 2001; Baumgartner *et al.*, 2007; Zita *et al.*, 2009; Chung & Lee, 2014). The reason why the whole egg weight for hens at the age of 21 weeks differ so much from the rest of the ages, is accredited to hens reaching maturity (Jacob *et al.*, 2003). This leads to an increased productivity, which is confirmed by the layer production curve as explained in Section 2.2.1 (Figure 2.1). Hens normally start laying eggs between the ages of 18 to 22 weeks. Six weeks after hens start laying eggs, their reproductive organs start to develop more, resulting in a sharp increase in egg productivity and egg weight (Jacob *et al.*, 2003) as seen in Figure 2.1. An additional reason for the sharp weight increase is that hens, which are older than 30 weeks, have a higher nutrient requirement than younger birds (Applegate & Angel, 2014) and can utilize nutrients more efficiently (Sifri, 1995). After 30 weeks, the increase in egg weight starts to gradually reach a plateau, which would explain the trend seen in Figure 3.3 (Jacob *et al.*, 2003).

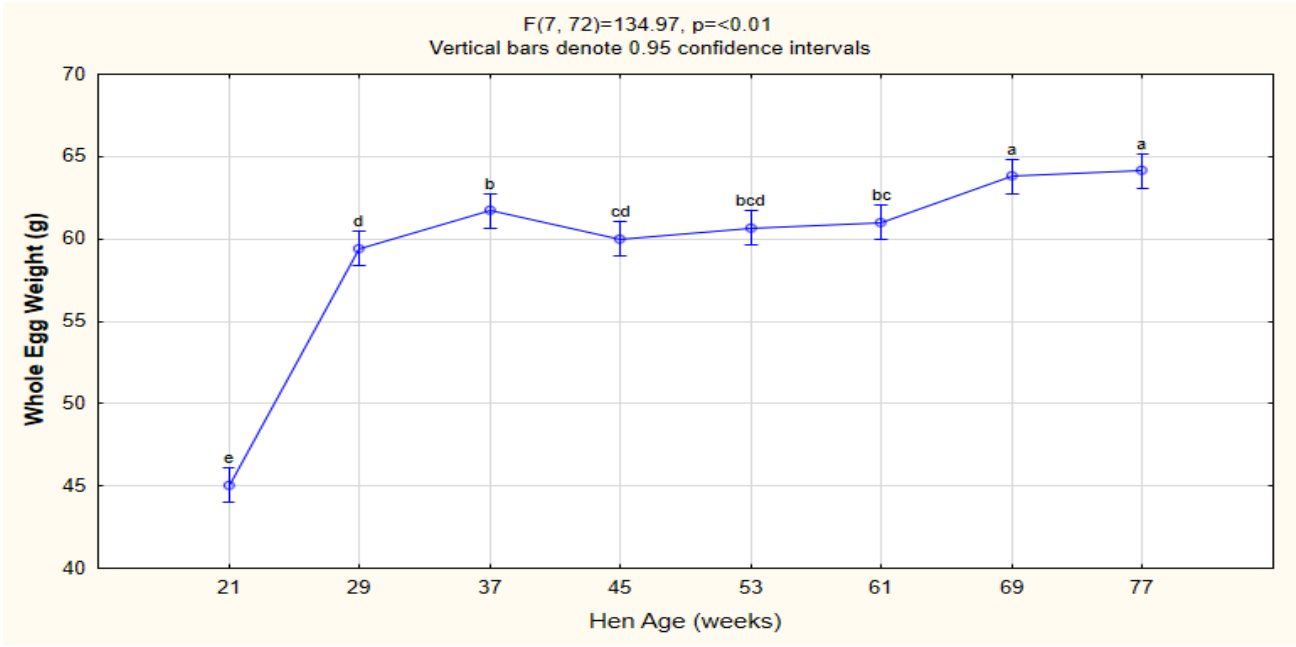
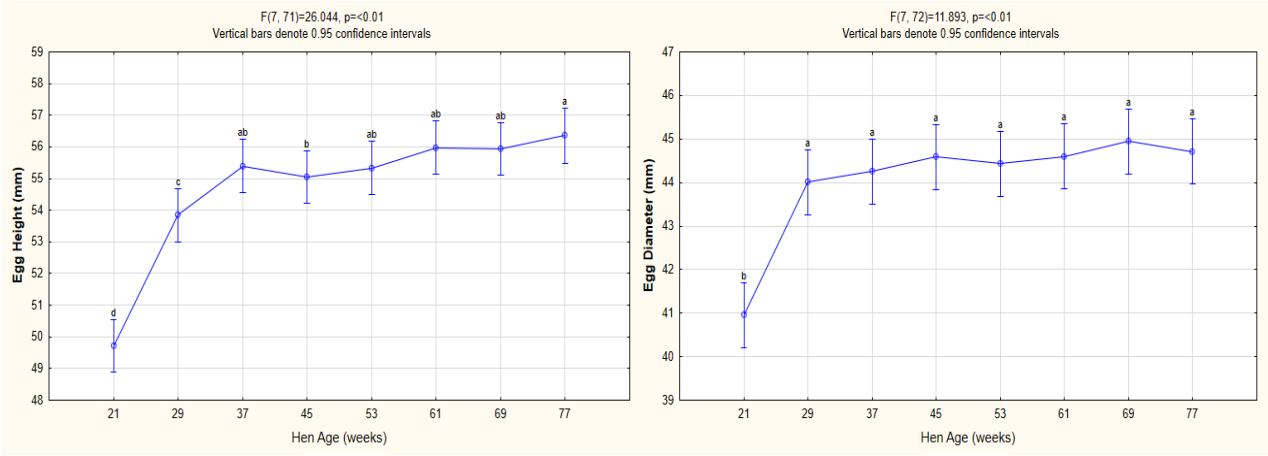


Figure 3.3 Graph showing the change in the mean whole egg weight (g) produced by Amberlink hens with ages ranging from 21 to 77 weeks.

Egg shape is usually described as the ratio of diameter to length (egg shape index), typically having a lower value at a young age and increasing as the hens get older. This is due to an elongation of the egg height and a less pronounced increase in egg diameter with increase in hen age (Baumgartner *et al.*, 2007). Both egg height ($P<0.01$) and egg diameter ($P<0.01$) was significantly influenced by hen age (Table 3.3). It was observed that egg height and diameter increased most between 21 and 29 weeks (Figure 3.4a and Figure 3.4b), respectively. Once again, the reason for this sharp increase can be attributed to the onset of egg production and development of the reproductive organs between 18 and 22 weeks of age. The same pattern was observed for whole egg weight between the ages of 21 and 29 weeks, as explained above. These results are comparable with the study done by Nikolova & Kocevski (2006), concluding that the egg height and diameter

increased with an increase in hen age, but length showed a greater increase than diameter. Similar results were obtained in various other studies over the years (Pavlovski *et al*., 1981; Orhan *et al*., 2002; Van Den Brand *et al*., 2004; Zita *et al*., 2009; Zita *et al*., 2013). However, the egg shape indexes all decreased in these studies with an increase in hen age, due to the elongation of the eggs. In the current study, the shape index also decreased between the ages of 21 and 37 weeks (0.82 to 0.79), but remained relatively the same between the ages of 37 and 77 weeks (0.79 to 0.79) which is similar to the findings of Altan *et al*. (1998). This result is due to a greater increase in egg height than egg diameter in eggs laid at an older age compared to eggs laid by younger hens. A study done by Krawczyk (2009) showed that egg height and diameter was influenced differently between six hen breeds with an increase in hen age. This suggests that the differences in egg height and diameter seen between the current study and the studies done by Pavlovski *et al*. (1981); Orhan *et al*, (2002); amongst others, can be ascribed to breed differences. However, no literature could be found for the shape index of eggs laid by different aged Amberlink hens, therefore the trend for this breed is uncertain. Additionally, this study was not designed to test breed differences, therefore this theory is considered ambiguous.



(a) **(b)**

Figure 3.4 Graph showing the change in (**a**) egg height (mm) and (**b**) egg diameter (mm) of eggs produced by Amberlink hens with ages ranging from 21 to 77 weeks.

Shell thickness and shell weight was significantly (P<0.01) influenced by hen age (Table 3.3). The increase in shell weight could be attributed to the increase in the whole egg weight, which led to an increase in egg height and diameter (Whitehead *et al.*, 1991; Silversides & Scott, 2001), which was confirmed in the current study. It was thus accepted that there was a positive correlation between shell weight and whole egg weight. This assumption was confirmed as a correlation coefficient of 0.78 was obtained between these two parameters. However, the increase in hen age led to a decrease in shell thickness of eggs. The decrease in shell thickness could possibly be caused by two factors. Firstly, as hens age, they lay larger eggs (increased egg content) with no proportionate increase in the calcium deposition. To accommodate the larger eggs, the shells become thinner (Roland *et al.*, 1975; Robert, 2004). Secondly, Hurwitz and Griminger (1962)

explained that the decrease in thickness could be due to older hens' inability to absorb calcium effectively by the small intestine and hens also mobilize less skeletal calcium compared to younger hens. This resulted in less calcium deposition during shell formation, which was also confirmed later by Clunies *et al.* (1992). However, the efficiency at which calcium was deposited and absorbed by different aged hens was not tested in the current study. The current results confirms the findings of other studies that have also shown, that an increase in hen age led to an increase in shell weight but also reported a reduction in the shell thickness of eggs laid by older hens (Suk & Park, 2001; Padhi *et al.*, 2013; Zita *et al*., 2013). It is observed in Table 3.3 that the greatest increase in shell weight was between the ages of 21 and 29 weeks of age. It is thus not surprising that the greatest reduction in shell thickness was between the ages of 21 and 37 weeks (Table 3.3). This result can once again be ascribed to the hen's increased production efficiency after maturity is reached (Jacob *et al.*, 2003; Kleyn, 2013).

Hen age had a significant effect ($P<0.01$) on the occurrence of bumps. During the current study, it was noted that eggs laid by older hens displayed a higher occurrence in shell bumps that eggs laid by younger hens. Eggs laid by younger hen ages such as 21, 29, 37 and 45 weeks of age had a 0%, 10%, 0% and 10% occurrence of shell bumps, respectively. While eggs laid by older hens which included 53, 61, 69 and 77 weeks of age had a 20%, 20%, 40% and 50% occurrence of shell bumps, respectively. These results are comparable with the study done by Akyurek and Okur (2009). They concluded that shell pimples or bumps have a higher tendency to occur in older hens due to a change in the calcium depositing process. Older hens' shell glands might deposit calcium unevenly around the surface of the interior content during shell formation (Akyurek & Okur, 2009; Tůmová *et al.*, 2014).

3.3.2 Effect of hen age on internal egg quality parameters

The results in Table 3.4 showed that the age of the hen significantly influenced ($P\leq0.05$) the yolk height, thick albumen height, thin albumen height, yolk weight, albumen weight, HU, thin albumen spread, yolk colour L*, yolk colour a* and the Roche yolk colour fan score. Hen age had no significant effect ($P>0.05$) on thick albumen spread, yolk colour b* and no significant effect on any of the categorical data. This included the occurrence (present or not present) of rupturing vitelline membranes, blood spots and meat spots. Categorical data, except for the Roche yolk colour fan, is not displayed in Table 3.4.

In Table 3.4 egg yolk height increased significantly between the ages of 21 to 77 weeks. Ahmet *et al*. (2009) found that as the hen's age increased from 28 to 80 weeks a decrease in the yolk index (yolk height to yolk diameter ratio) was observed. This result could be due to a substantially larger increase in the yolk diameter compared to the yolk height. This could support the results obtained in the current research trial, but it cannot be said for certain as only the yolk index was indicated and not the yolk height. In a study done by Rabie *et al*. (1997) it was shown that egg yolk height increased from the age of 65 weeks to 73 weeks due to an increase in total egg size,

Table 3.4 The mean ± standard deviation of internal egg quality parameters for eggs laid by Amberlink hens with ages ranging from 21 to 77 weeks

Parameter		Yolk Height (mm)	Thick Albumen Height (mm)	Thin Albumen Height (mm)	Yolk Weight (g)	Albumen Weight (g)	Haugh Unit (HU)
Treatment (Hen Ages in Weeks)	21	18.51^{d} ± 0.59	9.69^{a} ± 0.51	2.85^{a} ± 0.22	11.07^{e} ± 0.76	27.92^{e} ± 1.41	101.37^{a} ± 1.85
	29	19.48^{c} ± 0.63	8.74^{b} ± 0.59	2.20^{b} ± 0.12	14.80^{cd} ± 0.86	35.86^{d} ± 1.12	92.48^{b} ± 1.36
	37	19.75^{c} ± 0.43	8.70^{b} ± 0.39	2.14^{b} ± 0.22	15.38^{cd} ± 0.41	36.02^{d} ± 1.21	92.17^{b} ± 1.87
	45	19.68^{c} ± 0.53	8.02^{c} ± 0.33	2.13^{b} ± 0.32	15.54^{c} ± 0.89	36.35^{cd} ± 1.07	89.82^{c} ± 1.48
	53	19.88^{bc} ± 0.39	8.02^{c} ± 0.28	1.89^{c} ± 0.32	16.24^{b} ± 0.66	36.44^{cd} ± 1.46	89.26^{c} ± 1.21
	61	19.73^{c} ± 0.52	8.03^{c} ± 0.35	1.86^{c} ± 0.10	16.33^{b} ± 0.82	37.05^{bc} ± 0.70	89.39^{c} ± 1.44
	69	20.21^{ab} ± 0.49	7.26^{d} ± 0.38	1.76^{cd} ± 0.11	16.71^{ab} ± 0.78	37.90^{ab} ± 0.69	87.02^{d} ± 1.39
	77	20.42^{a} ± 0.38	7.59^{d} ± 0.23	1.64^{d} ± 0.17	17.08^{a} ± 0.69	38.66^{a} ± 1.09	86.01^{d} ± 1.14
	P value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Parameter		Thick Albumen Spread (mm)	Thin Albumen Spread (mm)	Yolk Colour L*	Yolk Colour a*	Yolk Colour b*	Roche Colour Fan
Treatment (Hen Ages in Weeks)	21	50.00 ± 0.00	124.00^{c} ± 34.62	49.79^{b} ± 1.66	15.68^{a} ± 0.82	46.85 ± 1.88	12.10^{a} ± 0.73
	29	57.00 ± 22.13	159.00^{b} ± 34.54	51.57^{a} ± 1.32	15.67^{a} ± 0.65	45.68 ± 1.83	11.90^{ab} ± 0.73
	37	50.00 ± 0.00	175.50^{ad} ± 21.66	51.54^{a} ± 1.73	14.90^{ab} ± 0.98	45.86 ± 1.78	11.40^{bc} ± 0.51
	45	50.00 ± 0.00	168.50^{ab} ± 35.67	51.69^{a} ± 1.63	14.52^{bc} ± 0.90	46.70 ± 2.01	11.50^{ab} ± 0.70
	53	64.00 ± 29.51	172.00^{ab} ± 30.47	51.72^{a} ± 1.83	13.77^{cd} ± 1.05	46.94 ± 1.52	11.70^{ab} ± 0.94
	61	57.00 ± 22.13	179.00^{ab} ± 8.43	51.66^{a} ± 1.21	13.93^{cd} ± 0.96	46.81 ± 1.70	10.80^{cd} ± 0.42
	69	64.00 ± 29.81	181.00^{ab} ± 9.66	51.83^{a} ± 1.43	13.18^{d} ± 0.81	46.74 ± 1.60	10.80^{cd} ± 0.63
	77	71.00 ± 33.81	183.00^{a} ± 10.32	52.05^{a} ± 1.16	13.21^{d} ± 0.92	46.07 ± 1.89	10.40^{d} ± 0.84
	P value	0.27	<0.01	0.05	<0.01	0.60	<0.01

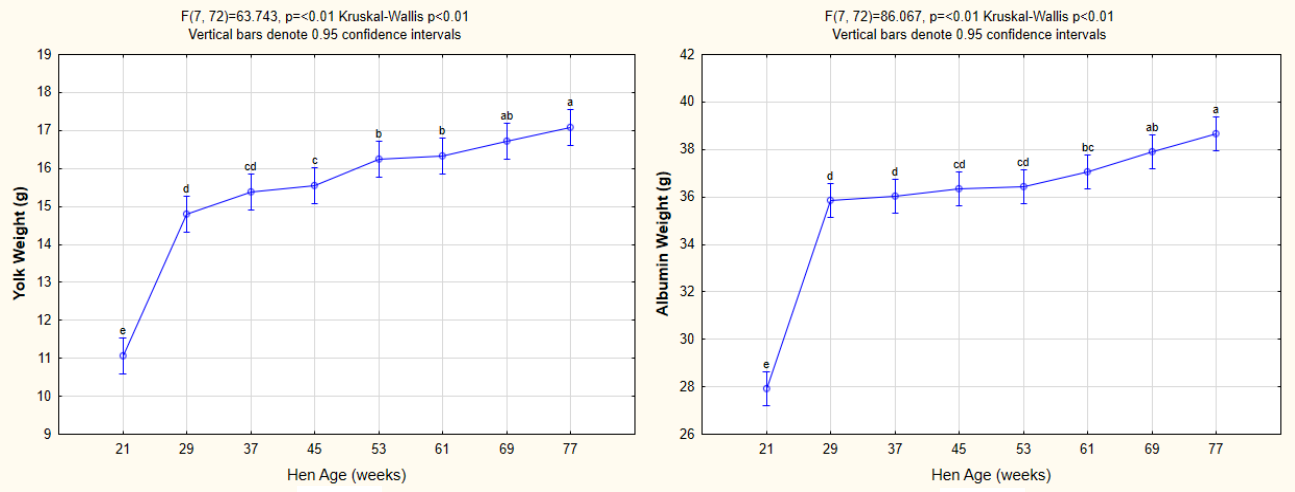
($^{a, b, c, d, e}$) Means with different superscripts within the same column differ significantly (P≤0.05).

which is comparable with the results obtained between the ages of 61 to 77 weeks in the current trial. In the current study the age of the hen when the eggs were laid did not have a significant effect on the occurrence of rupturing egg yolks, meaning the vitelline membrane kept its structural integrity. It is thus possible to assume that the vitelline membrane kept the yolks form, ensuring the yolk height does not decrease, which occurs during storage due to loss in the structural integrity of the vitelline membrane (Brake *et al.*, 1997).

Albumen quality declines with an increase in bird age (Roberts & Ball, 2005; Silversides & Scott, 2001). The albumen consists of the thick and thin albumen layers. The thick albumen height displays significant changes during the hen's production cycle. The thin albumen height decreased as well, but changes are not as pronounced as observed for the thick albumen height (Gennadios *et al.*, 1998; Curtis *et al.,* 2005). As the hen ages, the thick and thin albumen height decreases, even though the albumen weight and total egg weight increases (Hill & Hall, 1980; Silversides & Scott, 2001). These results coincides with the results obtained in the current study as thick and thin albumen height significantly ($P<0.01$; Table 3.4) decreased, yet egg weight (Table 3.3) and albumen weight (Table 3.4) increased as the hen aged. The albumen consist of various proteins, but the most important proteins which determine the height of the thick and thin albumen, is ovomucin (Toussant & Latshaw, 1999) and its interaction with the lysozyme protein (Lewko & Gornowicz, 2009). The gel like structure of the thick and thin albumen is dependent on the stability of the lysozyme- ovomucin complex. The α-ovomucin protein is covalently linked to lysozyme with disulphide bonds (Hammershøj & Qvist, 2001). However, Hayakawa *et al.* (1983) explained that α-ovomucin in eggs laid by older hens, have a higher tendency to be released from the lysozyme-ovomucin complex by unknown mechanisms. It is reported that the released ovomucin is solubilised into a liquid fraction of the albumen, which contribute to the thinning of the egg albumen and leads to the reduction in the thick and thin albumen height. This would explain the reduction in the thick and thin albumen height observed in Table 3.4.The results from the current study are comparable with the study done by Leeson and Caston (1997). Their results indicated that thick and thin albumen height were higher in younger hens (22 weeks) when compared to older hens (66 weeks), which was also later confirmed by Rajkumar *et al.* (2009).

Both yolk and albumen weight increased significantly ($P<0.01$) with the biggest change in weight occurring between 21 and 29 weeks of age (Figure 3.5). The differences seen in yolk and albumen weight observed between different hen ages (Table 3.4) can once again be attributed to the increase of the whole egg weight (Hill & Hall, 1980; Silversides, 1994; Silversides & Scott, 2001). Since the yolk weight and albumen weight are directly correlated to total egg weight, these two components increase proportionately as the egg weight increases (Flethcer *et al.*, 1983; Hussein *et al.*, 1993). This statement is confirmed in the current study as egg weight was highly correlated with yolk weight (0.92) and albumen weight (0.93). Various other studies found similar results, confirming that higher yolk and

albumen weights can be associated with an increase in hen age (Burley & Vadehra, 1989; Kaminska & Skraba, 1991; Hussein *et al*., 1993; Rossi & Pompei, 1995; Applegate *et al*., 1998). However, Chung and Lee (2014) did not report a significant difference between the yolk weight nor the albumen weight as hens aged. This could be attributed to their use of a narrow hen age interval ranging from 40 to 60 weeks, which coincides with a period that showed small differences in the current study. The age interval used by Chung and Lee (2014) was not a good range to represent the hen's egg productivity period, which could be a reason why no significant differences were noticed in yolk weight and albumen weight. It was also noted that the yolk to albumen weight ratio increased with an increase in hen age in the current study. The yolk to albumen weight ratio increased from 0.39 (21 weeks of age) to 0.44 (77 weeks of age), which suggest that there was a greater increase in yolk weight than albumen weight. This result coincides with the study done by Hussein *et al.* (1993), reporting a higher yolk to albumen weight ratio value of 0.39 in eggs laid by older hens (58 weeks of age) compared to a ratio of 0.37 observed in younger hens (32 weeks of age). The difference in the yolk to albumen ratio in their study might not be as pronounced as the difference observed in the current study, but could be attributed to the use of a narrower age interval by Hussein *et al.* (1993) compared to the current study (21 to 77 weeks of age).



(a) **(b)**

Figure 3.5 Graph showing the change in (**a**) yolk weight (g) and (**b**) albumen weight (g) of eggs produced by Amberlink hens with ages ranging from 21 to 77 weeks.

Analysis of the HU indicated that hen age had a significant effect on this parameter (P<0.01; Table 3.4). Since HU is correlated to the thick albumen height and whole egg weight, it can be expected that changes in these two factors would influence the HU. If the thick albumen height decreases, a decrease in the HU will be observed, while an increase in whole egg weight will lead to a decrease in HU and *vice versa* (Silverside, 1977; Silversides & Scott, 2001; Williams, 1992). This statement is confirmed as HU was calculated by using a logarithm of the thick albumen height, adjusted to the whole

egg weight (Haugh, 1937), as seen in Equation 3.2. The significant change can thus be ascribed to the increase in whole egg weight (Table 3.3) and decrease thick albumen height (Table 3.4), as HU and the whole egg weight showed a high negative correlation (r = -0.86), while HU and the thick albumen height showed a highly positive correlation (r = 0.88) in the current study. The youngest hen age group (21 weeks) laid eggs with the highest HU, but as the age progressed, HU decreased (Figure 3.6). The biggest difference in HU was between the age of 21 and 29 weeks of age and can be ascribed to the substantial decrease in thick albumen height noticed in eggs laid by hens aged between 21 and 29 weeks (Figure 3.6). The current study's result is in accordance with numerous studies done over the years to test the effect of hen age on HU (Akbaş *et al*., 1996; Kirunda & McKee, 2000; Ledur *et al.*, 2002; Curtis *et al*., 2005; Manley *et al.*, 2009; Salajegheh *et al.*, 2012). Petek *et al.* (2009) on the other hand showed no significant differences in HU for eggs collected from hens between the ages of 24 to 36 weeks, but in the current study big differences were noticed. This can once again be ascribed to an age interval too narrow to adequately test the effect of hen age on HU (Haugh, 1937; Akbaş *et al.*, 1996). When observing Figure 3.6 it can be concluded that all of the eggs can be classified as an AA grade (HU>72). All of the eggs can be classified as "fresh" as well, considering that all the eggs used in the current trial had a HU value above 60 (Zhao *et al.*, 2010).

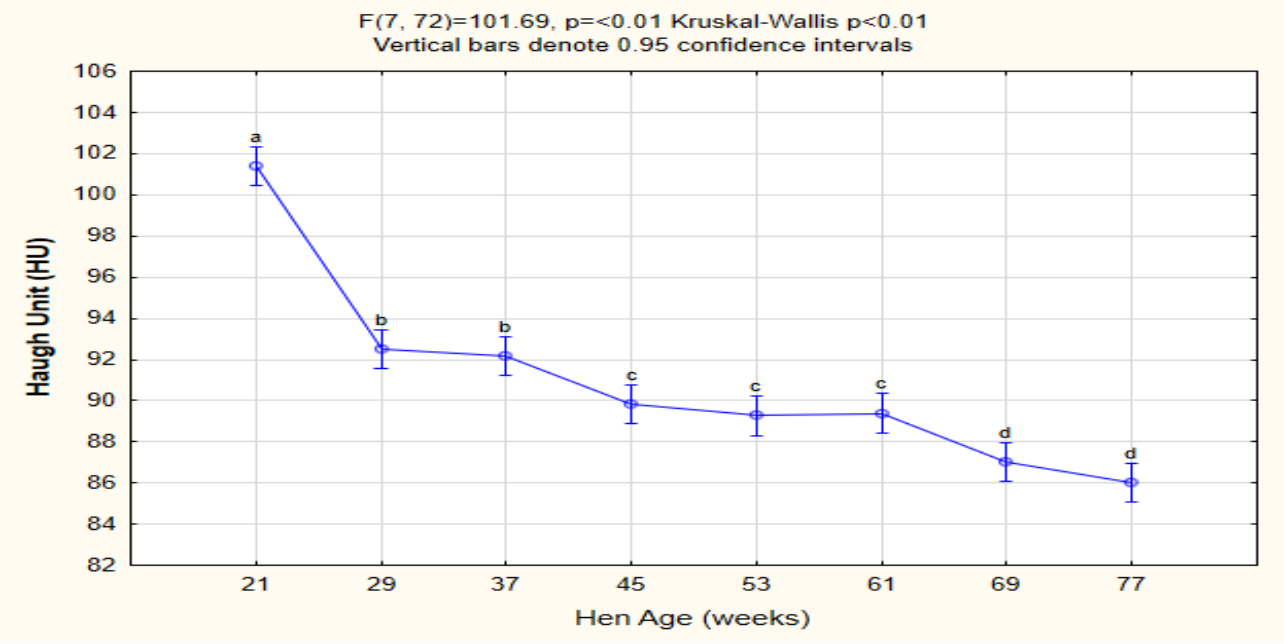


Figure 3.6 Graph showing the change in the Haugh Unit (HU) of eggs produced by Amberlink hens with ages ranging from 21 to 77 weeks.

Thin albumen spread was significantly influenced (P<0.01) by hen age and thick albumen spreading showed an increase in the spreading distance, nonetheless it was not significant (P = 0.27) as seen in Table 3.4. Some mean values of the thick and thin albumen spreading distance in Table 3.4 show high standard deviation, which is due to the assignment of categories. For example, if

an age group (ten eggs) contained nine eggs assigned to category one and one egg assigned to category two, it would lead to high standard deviations between egg samples of that age group, due to big differences in the distance (mm) between categories. Eggs from 21-week-old hens displayed the shortest spreading distance. The increased spreading of the thin albumen is directly related to increase in the size of the egg (egg weight), more specifically the albumen weight, as suggested by Leeson and Caston (1997) which was also later confirmed by Robert (2004). This statement could not be confirmed in the current study as the albumen weight was only moderately correlated with the thin albumen spread ($r = 0.62$). It is possible that the alteration in the lysozyme-ovomucin complex, as discussed previously, could have led to increased spreading of the thin albumen, but this could not be concluded for certain. Leeson and Caston (1997) reported a higher albumen area in eggs laid by 66-week-old hens (115.6 cm^2) compared to eggs laid by 22-week-old hens (85.7 cm^2), which is comparable with the current study as the radius (distance from the yolk to the furthest point of the albumen) increased from 124 mm at 21 weeks of age to 183 mm at 77 weeks of age (Table 3.4).

Colour b* showed no significant differences ($P = 0.60$) between ages. However, both colour L* ($P = 0.05$) and colour a* ($P<0.01$) showed significant differences (Table 3.4). Colour L* (lightness) increased and a* (redness) decreased with an increase in hen age, which could possibly be attributed to a decrease in the level of carotenoids in the yolk as hens get older as suggested by Cherian, (2008). The Roche colour fan score was significantly reduced ($P<0.01$) with an increase in hen age (Table 3.4). The increase in lightness (L*) is confirmed by the Roche colour fan score, also displaying higher scores (darker yolks) in eggs laid by younger hens compared to eggs laid by older hens, displaying lower colour fan scores (lighter or paler yolks). Yolk colour is influenced by the level of carotenoids, also known as xanthophyll pigments, which are obtained from the hen's diet (Karunajeewa *et al.*, 1984; Chung & Lee, 2014). The carotenoids in the yolk provide antioxidant protection during embryo development and hatching, which is a period characterized by high oxidative stress (Surai *et al.*, 1999). Carotenoids are absorbed through passive diffusion across the microvilli of the mucosal epithelium present in the small intestine (mostly jejunum) (Cohn, 1997) and delivered to the liver by portomicrons (lipoproteins) and then incorporated into the yolk (Surai *et al.*, 2001). Studies have shown, that the reduction in yolk colour in eggs as the hen ages, can be attributed to a decrease in carotenoid absorption from the small intestinal track resulting in reduced deposition of carotenoids in the yolk (Cherian, 2008; Surai *et al.*, 2016). Fischer Da Silva *et al.* (2007) concluded in his study that villi density in the small intestine decreased with an increase in hen age. It is thus possible that the increase in lightness and decrease in redness as well as the decrease in the Roche yolk colour fan scores is due to less carotenoid absorption by older hens than younger ones. It is also possible that the increase in yolk lightness with increase in hen age is due to a higher reflectance by the spectrophotometer as L* is defined as the reflectance of light by an object (Nys, 2000). It is already established that egg yolk weight increased with an increase

in hen age. It is thus possible that older hens lay eggs with thinner vitelline membranes, which could lead to more light reflection compared to younger hens' egg yolks. However, the decrease in redness and the Roche yolk colour fan scores suggest a decrease in colour, which is likely linked to the carotenoid content of the yolk. Roberson *et al.* (2005) also found that eggs laid by older hens showed higher yolk colour L* values when compared to younger hens and Minelli *et al.* (2007) also reported a significant decrease in colour a* with an increase in hen age.

3.3.3 Effect of hen age on proximate composition

The hens' age had a significant effect (P≤0.05) on the proximate composition of eggs, except for the ash content (P = 0.99) (Table 3.5). Protein, lipid and ash content are displayed on a DM basis (Table 3.5).

Table 3.5 The means ± standard deviations of proximate composition for eggs laid by Amberlink hens with ages ranging from 21 to 77 weeks

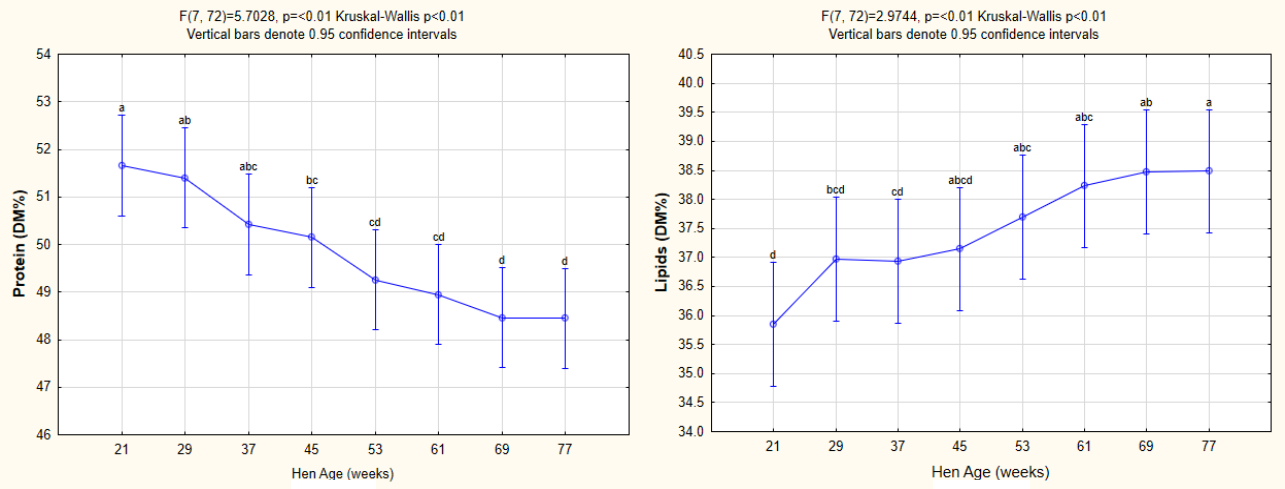
Parameter			Dry Matter (DM) Basis		
		Moisture (%)	Protein (%)	Lipids (%)	Ash (%)
Treatment (Hen Ages in Weeks)	**21**	76.62^{a} ± 1.55	51.65^{a} ± 1.48	35.84^{d} ± 1.43	3.55 ± 0.29
	29	75.48^{ab} ± 1.63	51.40^{ab} ± 1.73	36.97^{bcd} ± 1.58	3.60 ± 0.28
	37	75.20^{ab} ± 1.66	50.41^{abc} ± 1.82	36.93^{cd} ± 1.55	3.52 ± 0.22
	45	74.14^{bc} ± 1.59	50.15^{bc} ± 1.26	37.14^{abcd} ± 1.99	3.54 ± 0.38
	53	73.57^{c} ± 1.46	49.26^{cd} ± 1.54	37.69^{abc} ± 1.74	3.55 ± 0.11
	61	73.59^{c} ± 1.83	48.95^{cd} ± 1.90	38.22^{abc} ± 1.78	3.54 ± 0.22
	69	73.16^{c} ± 1.70	48.46^{d} ± 1.62	38.47^{ab} ± 1.74	3.47 ± 0.38
	77	73.09^{c} ± 1.79	48.44^{d} ± 1.86	38.48^{b} ± 1.59	3.56 ± 0.29
	P value	<0.01	<0.01	<0.01	0.99

($^{a, b, c, d}$) Means with different superscripts within the same column differ significantly (P≤0.05).

The moisture content of the eggs were significantly (P<0.01) affected by increasing hen age (Table 3.5). Applegate *et al.* (1998) concluded that moisture, if expressed as a percentage of whole egg weight, was higher in eggs laid by younger birds (36 weeks) compared to eggs laid by older hens (55 weeks). This is due to an increase in the solid content of eggs with an increase in hen age (Flethcer *et al.*, 1983). Protein is the main contributing solid component in the albumen and lipids the main contributing solid component in the yolk of eggs (Ahn *et al.*, 1997). Rose *et al.* (1966) reported an increase in the solid content in eggs, primarily due to an increase in the yolk (lipid content) with an increase in hen age, however the moisture content was not indicated. Ahn *et al.* (1997) later reported lower solid with higher moisture content in eggs laid by younger hens (28 weeks of age) and higher solid with lower moisture content in eggs laid by older hens (55 to 78 weeks of age). These results coincide

with the current study explaining the decrease in moisture content and the increase in lipid content of eggs with increase in hen age, as seen in Table 3.5.

The protein content decreased significantly ($P<0.01$) when hen age increased (Table 3.5). The lipid content of eggs on the other hand, significantly ($P<0.01$) increased with an increase in the age of the hen (Table 3.5). It is important to note that the decrease in protein and increase in lipid content is expressed as a percentage of whole egg weight. The albumen consist mostly out of protein while the yolk consists of lipids and protein, with the majority being contributed by the lipids (Stadelman & Cotterill, 1977; OnbaŞılar *et al.*, 2011). The ratio between the yolk and albumen is greatly influenced by the age of the hen, which therefore impacts the protein to lipid ratio (Flethcer *et al.*, 1983). As the hen ages, the total egg weight increases and at the same time there is a greater proportional increase in the yolk weight compared to the albumen weight (Washburn, 1979) which was also observed in the current study. It can thus be concluded that the lipid to protein ratio is less in younger hens compared to older ones, which was also reported by (Kaminska & Skraba, 1991). This trend is depicted in Figure 3.7a and Figure 3.7b, where protein content decreased and lipid content increased respectively, as hen age increased. Cotterill *et al.* (1977) made the assumption that the protein content is negatively correlated with the lipid content as hen age increased. This assumption was confirmed in the current study as well with a strong negatively correlated relationship ($r = -0.90$) between protein and lipid content. The study by Ahn *et al.* (1997) also found that eggs laid by 78-week-old hens had a higher yolk to albumen ratio and thus a higher lipid to protein ratio compared to eggs laid by 28-week-old hens. It is thus likely that as hens age, more lipids are deposited in the yolk and less protein in the albumen due to an increase in the yolk size and a reduction in albumen size, which would explain the results in Table 3.5 and Figure 3.7. However, protein and lipids both increased on a wet basis (liquid egg) as seen in Chapter 5 (Section 5.3.2.1).



(a) **(b)**

Figure 3.7 Graph showing the change in dry matter content of (**a**) protein (%) and (**b**) lipid (%) of eggs produced by Amberlink hens with ages ranging from 21 to 77 weeks.

3.4 Conclusion

The aim of the study was to evaluate the effect of hen age on certain egg quality parameters. It can be concluded that hen age had a significant effect on all the external egg quality parameters, except on the occurrence of banded shelled eggs. Egg weight, egg height, egg diameter, shell thickness, shell weight and bumps were significantly influenced by hen age. The increase in egg weight (size) resulted in an increase in egg height, diameter and shell weight. However, shell thickness decreased, which could have been the result of numerous factors. The biggest change in egg weight, egg height, egg diameter, shell thickness and shell weight occurred between the age of 21 and 29 weeks of age. This result was ascribed to hens reaching maturity, which increased development of their reproductive organs, leading to higher egg weights which directly impacted the other external quality parameters.

Hen age also led to significant changes in the majority of the internal egg quality parameters. The increase in egg weight (size) led to an obvious increase in the egg yolk height, yolk weight and albumen weight. However, thick albumen height and thin albumen height decreased likely due to alteration in the lysozyme-ovomucin complex. The albumen therefore decreased in viscosity, which could also explain the increase of the thin albumen spreading distance. However, this could be due to an increase in albumen weight. The decrease in HU was caused by the decrease in thick albumen height as HU is expressed as the logarithm of thick albumen height adjusted to egg weight. As hen age increased, eggs displayed lighter (paler) egg yolks, which was confirmed by the Roche yolk colour fan and the L* colour value. Yolk colour a* (redness) showed a significant reduction with no effect on yolk colour b* (yellowness). It was concluded that the decrease in colour, with an increase in hen age was likely due to a decreased absorption of carotenoids responsible for colour. Noteworthy changes occurred in the majority of the quality parameters between the ages of 21 and 29 weeks of age. This occurrence can

be ascribed to hens reaching maturity and increased reproductive performance. It might thus be beneficial for future studies to investigate egg quality changes between younger ages.

Hen age also had a significant effect on all the proximate characteristics except the ash content. Increased hen age led to a decrease in the moisture (%) content likely due to an increase in the solid content of eggs. This was consistent with the lipid content, as lipids were higher in older hens than younger ones. Protein content on the other hand decreased. It was concluded that older hens lay eggs with a higher lipid content and reduced protein content as these two parameters are negatively correlated.

Hen age significantly affected all the external quality parameters (six), ten out of the eighteen internal quality parameters and three out of the four proximate parameters. Therefore, it can be concluded that hen age is an important factor to consider when assessing egg quality due to its substantial effect on most egg quality parameters.

3.5 References

Abd-Elhady, M. 2014. Effect of citric acid, calcium lactate and low temperature prefreezing treatment on the quality of frozen strawberry. Ann. Agric. Sci. 59, 69–75.

Abdelhamid, A. M., & Dorra, T. M. 1990. Study on effects of feeding laying hens on separate mycotoxins (aflatoxins, patulin, or citrinin)-contaminated diets on the egg quality and tissue constituents. Arch. Tierernahr. 40, 305–316.

Abrahamsson, P., & Tauson, R. 1995. Aviary systems and conventional cages for laying hens. Acta Agric. Scand. A Anim. Sci. 45, 191–203.

Ahn, D. U., Kim, S. M., & Shu, H. 1997. Effect of egg size and strain and age of hens on the solids content of chicken eggs. Poult. Sci. 76, 914–919.

Akbaş, Y., Altan, Ö., & Koçak, Ç. 1996. Effects of hen's age on external and internal egg quality characteristics. Turkish J. Vet. Anim. Sci. 20, 455–460.

Akter, Y., Kasim, A., Omar, H., & Sazili, A. Q. 2014. Effect of storage time and temperature on the quality characteristics of chicken eggs. J. Food, Agric. Environ. 12, 87–92.

Akyurek, H., & Okur, A. A. 2009. Effect of storage time, temperature and hen age on egg quality in free-range layer hens. J. Anim. Vet. Adv. 8, 1953–1958.

Al-Shami, M. A., Salih, M. E., & Abbas, T. E. 2012. Effects of dietary inclusion of alfalfa (Medicago sativa L.) leaf meal and Xylam enzyme on laying hens' performance and egg quality. Res. Opin. Anim. Vet. Sci. 2, 14–18.

Altan, Ö., Oğuz, I., & Akbaş, Y. 1998. Effects of selection for high body weight and age of hen on egg characteristics in Japanese quail (coturnix coturnix japonica). Turkish J. Vet. Anim. Sci. 22, 467–473.

AOAC. 2002. Association of Official Analytical Chemists (AOAC) International. fficial methods Anal. AOAC Int. 17th ed. Arlington, Virginia, USA.

Applegate, T. J., & Angel, R. 2014. Nutrient requirements of poultry publication: History and need for an update. J. Appl. Poult. Res. 23, 567–575.

Applegate, T. J., Harper, D., & Lilburn, M. S. 1998. Effect of hen production age on egg composition and embryo development in commercial pekin ducks. Poult. Sci.

Bacon, W. L., & Skala, J. H. 1968. Ovarian follicular growth and maturation in laying hens and the relation to egg quality. Poult. Sci. 47, 1437–1442.

Bar Arie, & Hurwitz Shmuel. 1987. Vitamin D metabolism and calbindin (calcium-binding protein) in aged laying hens. , 1775–1779.

Bar, A., Razaphkovsky, V., & Vax, E. 2002. Re-evaluation of calcium and phosphorus requirements in aged laying hens. Br. Poult. Sci. 43, 261–269.

Baumgartner, J., Končeková, Z., & Benková, J. 2007. Line Effect and phenotypic correlations among egg qualitative traits in japanese quail eggs selected on yolk cholesterol content. 2007, 13–18.

Beardsworth, P. M., & Hernandez, J.-M. 2004. Yolk colour - An important egg quality attribute. Int. Poult. Prod. 12, 17–18.

Bejaei, M., Wiseman, K., & Cheng, K. M. 2011. Influences of demographic characteristics, attitudes, and preferences of consumers on table egg consumption in British Columbia, Canada. Poult. Sci. 90, 1088–1095.

Bozkurt, Z., & Tekerli, M. 2009. The effects of hen age, genotype, period and temperature of storage on egg quality. Kafkas Univ. Vet. Fak. Derg.

Brake, J., Walsh, T., Benton, C., & Petitte, J. 1997. Egg handling and storage. Poult. Sci. 76, 144–151.

Van Den Brand, H., Parmentier, H. K., & Kemp, B. 2004. Effects of housing system (outdoor vs cages) and age of laying hens on egg characteristics. Br. Poult. Sci. 45, 745–752.

Britton, W. M. 1977. Shell membranes of eggs differing in shell quality from young and old hens. Poult. Sci. 56, 647–653.

Burley, R. W., & Vadehra, D. V. 1989. The avian egg : chemistry and biology. Wiley.

Burmester, B. R., & Card, L. E. 1937. On the nature of ‘meat spots’ in eggs. dep. anim. husbandry, Univ. Illinois, Urbana, Illinois, 235–239.

Castellini, C., Perella, F., Mugnai, C., & dal Bosco, A. 2002. Welfare, productivity and qualitative traits of egg in laying hens reared under different rearing systems. Photochem. Photobiol., 1–6.

Cherian, G. 2008. Egg quality and yolk polyunsaturated fatty acid status in relation to broiler breeder hen age and dietary n-3 Oils. Poult. Sci. 87, 1131–1137.

Chung, S. H., & Lee, K. W. 2014. Effect of hen age, storage duration and temperature on egg quality in

laying hens. Int. J. Poult. Sci. 13, 634–636.

Clunies, M., Parks, D., & Leeson, S. 1992. Calcium and phosphorus metabolism and eggshell formation of hens fed different amounts of calcium. Poult. Sci. 71, 482–489.

Cohn, W. 1997. Bioavailability of vitamin E. European Journal of Clinical Nutrition, 80–85.

Cook, F., & Briggs, G. 1986. The Nutritive value of eggs. Egg Sci. Technol., 141–63.

Cotterill, O. J., Marion, W. W., & Naber, E. C. 1977. A nutrient re-evaluation of shell eggs. Poult. Sci. 56, 1927–1934.

Curtis, P. A., Kerth, L. K., & Anderson, K. E. 2005. Quality and compositional characteristics of layer hens as affected by bird age.

Daniel, M., & Balnave, D. 1981. Responses of laying hens to gradual and abrupt increases in ambient temperature and humidity. Aust. J. Exp. Agric. 21, 189–195.

Dickerman, R. W., & Bahr, J. M. 1989. Molt induced by gonadotropin-releasing hormone agonist as a model for studying endocrine mechanisms of molting in laying hens 1. Poult. Sci. 68, 1402–1408.

Dvořák, P., Suchý, P., Straková, E., & Doležalová, J. 2010. Yolk color change depending on hen breeding technology. Acta Vet. Brno 79.

Fischer Da Silva, A. V., Maiorka, A., Borges, S. A., Santin, E., Boleli, I. C., & Macari, M. 2007. Surface area of the tip of the enterocytes in small intestine mucosa of broilers submitted to early feed restriction and supplemented with glutamine. Int. J. Poult. Sci. 6, 31–35.

Flethcer, D. L., Britton, W. M., Pesti, G. M., Rahn, A. P., & Savage, S. I. 1983. The relationship of layer flock age and egg weight on egg component yields and solids content. Poult. Sci. 62, 1800–1805.

Gennadios, A., Handa, A., Froning, G. W., Weller, C. L., & Hanna, M. A. 1998. Physical properties of egg white-dialdehyde starch films †. J. Agric. Food Chem 46, 1297–1302.

Grashorn, M. 2016. Effects of storage conditions on egg quality. 50, 23–25.

Hammershøj, M., & Qvist, K. B. 2001. Importance of hen age and egg storage time for egg albumen foaming. LWT - Food Sci. Technol. 34, 118–120.

Haugh, R. . 1937. The Haugh unit of measuring egg quality. US Poult. Mag 43, 522–555, 572–573.

Hayakawa, S., Kondo, H., Nakamura, R., & Sato, Y. 1983. Effect of β-ovomucin on the solubility of α-ovomucin and further inspection of the structure of ovomucin complex in thick egg white. Agric. Biol. Chem. 47, 815–820.

Hill, A. T., & Hall, J. W. 1980. Effects of various combinations of oil spraying, washing, sanitizing, storage time, strain, and age of layer upon albumen quality changes in storage and minimum sample sizes required for their measurement. Poult. Sci. 59, 2237–2242.

Hunter, R. 1958. Photoelectric color difference meter*. J. Opt. Soc. Am. 48, 985.

HunterLab. 2020. User Manuals for HunterLab Products.

Hurwitz, S., & Griminger, P. 1962. Estimation of calcium and phosphorus requirement in laying hens by balance techniques. J. Sci. Food Agric. 13, 185–191.

Hussein, S. M., Harms, R. H., & Janky, D. M. 1993. Research note: effect of age on the yolk to albumen ratio in chicken eggs. Poult. Sci. 72, 594–597.

Incharoen, T., Incharoen, T., & Yamauchi, K. 2009. Production performance, egg quality and intestinal histology in laying hens fed dietary dried fermented ginger. Int. J. Poult. Sci. 8, 1078–1085.

Jacob, J. P., Wilson, H. R., Miles, R. D., Butcher, G. D., & Mather, F. B. 2003. Factors affecting egg production in backyard chicken flocks. EDIS Publ. Syst., 1–8.

Jeffrey, F. P. 1945. Blood and meat spots in chicken eggs. Poult. Sci., 363–374.

Jones, D. B. 1931. Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins.

Kamanli, S., Demir, S., Kamanli, S., Durmus, I., Demir, S., & Tarim, B. 2015. Effect of different light sources on performance and egg quality traits in laying hens. Europ.Poult.Sci 79, 1612–9199.

Kaminska, B. Z., & Skraba, B. 1991. Analysis of hen types considering albumen: Yolk ratio and its change during the laying cycle. Natl. Res. Inst. Anim. Prod. Dep. Nutr. Physiol. Sarego 2, 43–45.

Karunajeewa, H., Hughes, R. J., Mcdonald, M. W., & Shenstone, F. S. 1984. A review of factors Influencing Pigmentation of Egg Yolks. Worlds. Poult. Sci. J. 40, 52–65.

Kato, A., Nakamura, R., & Sato, Y. 1970. Studies on changes in stored shell eggs. nihon chikusan gakkaiho 34, 1009–1013.

Kirunda, D. F. K., & McKee, S. R. 2000. Relating quality characteristics of aged eggs and fresh eggs to vitelline membrane strength as determined by a texture analyzer. Poult. Sci. 79, 1189–1193.

Kleyn, R. 2013. Chicken nutrition: A guide for nutritions and poultry professionals.

Krawczyk, J. 2009. Effect of layer age and egg production level on changes in quality traits of eggs from hens of conservation breeds and commercial hybrids. Ann. Anim. Sci. 9, 185–193.

Layman, K., & Rodriguez, R. 2009. Egg protein as a source of power, strength, and energy. Nutr. Today 44, 43–48.

Ledur, M. C., Liljedahl, L. E., McMillan, I., Asselstine, L., & Fairfull, R. W. 2002. Genetic effects of aging on egg quality traits in the first laying cycle of White Leghorn strains and strain crosses. Poult. Sci. 81, 1439–1447.

Leeson, S., & Caston, L. J. 1997. A Problem with characteristics of the thin albumen in laying hens. Poult. Sci. 76, 1332–1336.

Leeson, S., Walker, J. P., & Summers, J. D. 1982. Performance of laying hens subjected to intermittent lighting initiated at 24 weeks of age. Poult. Sci. 61, 567–568.

Lewko, L., & Gornowicz, E. 2009. Egg albumen quality as affected by bird origin. J. Cent. Eur. Agric. 10,

455–464.

Manley, M., Williams, P., Nilsson, D., & Geladi, P. 2009. Near infrared hyperspectral imaging for the evaluation of endosperm texture in whole yellow maize (Zea maize L.) Kernels. J. Agric. Food Chem. 57, 8761–8769.

Meehan, J. J., Sugihara, T. F., & Kline, L. 1962. Relationships between shell egg handling factors and egg product properties. Poult. Sci. 41, 892–900.

Mench, J. A., van Tienhoven, A., Marsh, J. A., McCormick, C. C., Cunningham, D. L., & Baker, R. C. 1986. Effects of cage and floor pen management on behavior, production, and physiological stress responses of laying hens. Poult. Sci. 65, 1058–1069.

Messens, W., Grijspeerdt, K., & Herman, L. 2005. Eggshell characteristics and penetration by Salmonella enterica serovar Enteritidis through the production period of a layer flock. Br. Poult. Sci. 46, 694–700.

Messens, W., Grijspeerdt, K., De Reu, K., De Ketelaere, B., Mertens, K., Bamelis, F., Kemps, B., De Baerdemaeker, J., Decuypere, E., & Herman, L. 2007. Eggshell penetration of various types of hens' eggs by Salmonella enterica serovar enteritidis. J. Food Prot. 70, 623–628.

Minelli, G., Sirri, F., Folegatti, E., Meluzzi, A., & Franchini, A. 2007. Egg quality traits of laying hens reared in organic and conventional systems. Ital. J. Anim. Sci. 6, 728–730.

Moscona, A. 1950. Studies of the egg of Bacillus libanicus (*Orthoptera, Phasmidae*) .

Nalbandov, A. V, & Card, L. E. 1943. The problem of blood clots and meat spots in chicken eggs. , 170–180.

Nikolova, N., & Kocevski, D. 2006. Forming egg shape index as influenced by ambient temperatures and age of hens. Biotechnol. Anim. Husb. 22, 119–125.

Nys, Y. 2000. Dietary carotenoids and egg yolk coloration - A review. Arch. fur Geflugelkd. 64, 45–54.

Ocak, N., Sarica, M., Erener, G., & Garipoglu, A. V. 2004. The Effect of body weight prior to molting in brown laying hens on egg yield and quality during second production cycle. Int. J. Poult. Sci. 3, 768–772.

OnbaŞılar, E. E., Erdem, E., Poyraz, O., & Yalcin, S. 2011. Effects of hen production cycle and egg weight on egg quality and composition, hatchability, duckling quality, and first-week body weight in Pekin ducks. Poult. Sci. 90, 2642–2647.

Orhan, H., Erensayin, C., & Aktan, S. 2002. Determining egg quality characteristics of Japanese quails (*Coturnix coturnix japonica*) at different ages. AGRIS since 42, 44–49.

Padhi, M. K., Chatterjee, R. N., Haunshi, S., & Rajkumar, U. 2013. Effect of age on egg quality in chicken. Indian J. Poult. Sci. 48, 122–125.

Patil, S. R., Cates, S., & Morales, R. 2005. Consumer food safety knowledge, practices, and demographic differences: Findings from a meta-analysis. J. Food Prot. 68, 1884–1894.

Pavlovski, Z., Masic, B., & Apostolov, N. 1981. Quality of eggs laid by hens on free range and in cages. Qual. Eggs Procedings First Eur. Symp. Apeldoom, Netherlands, 231–235.

Petek, M., Alpay, F., Gezen, S. S., & Çibik, R. 2009. Effects of housing system and age on early stage egg production and quality in commercial laying hens. Kafkas Univ. Vet. Fak. Derg. 15, 57–62.

Quentin, M., Bouvarel, I., & Picard, M. 2004. Short- and long-term effects of feed form on fast- and slow-growing broilers.

Rabie, M. H., SziláGyi, M., & Gippert, T. 1997. Effects of dietary L-carnitine on the performance and egg quality of laying hens from 65–73 weeks of age. Br. J. Nutr. 78, 615–623.

Rabon, H. W., Roland, D. A., Bryant, M., Barnes, D. G., & Laurent, S. M. 1990. Influence of sodium zeolite a with and without pullet-sized limestone or oyster shell on eggshell quality 1. Poult. Sci. 70, 1943–1947.

Rajkumar, U., Sharma, R. P., Rajaravind, K. S., Niranjan, M., Reddy, B. L. N., Bhattachar, T. K., & Chatterjee, R. N. 2009. Effect of genotype and age on egg quality traits in naked neck chicken under tropical climate from India. Int. J. Poult. Sci. 8, 1151–1155.

Rizzi, L., Simioli, M., Roncada, P., & Zaghini, A. 2003. Aflatoxin B1 and clinoptilolite in feed for laying hens: Effects on egg quality, mycotoxin residues in livers, and hepatic mixed-function oxygenase activities. J. Food Prot. 66, 860–865.

Roberson, K. D., Kalbfleisch, J. L., Pan, W., & Charbeneau, R. A. 2005. Effect of corn distiller's dried grains with solubles at various levels on performance of laying hens and egg yolk color. Int. J. Poult. Sci. 4, 44–51.

Robert, J. R. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. J. Poult. Sci. 41, 161–177.

Roberts, J. 2005. Egg quality guidelines for the Australian egg industry. World's Poult. Sci. Assoc., 23–26.

Roberts, J., Roberts, : J R, & Ball, W. 2005. Egg quality guidelines for the Australian egg industry. World's Poult. Sci. Assoc. 3, 23–26.

Rodriguez-Navarro, A., Kalin, O., Nys, Y., & Garcia-Ruiz, J. M. 2002. Influence of the microstructure on the shell strength of eggs laid by hens of different ages. Br. Poult. Sci. 43, 395–403.

Roland, D. A. 1979. Factors influencing shell quality of aging hens. Poult. Sci.

Roland, D. A., Sloan, D. R., & Harms, R. H. 1975. The ability of hens to maintain calcium deposition in the egg shell and egg yolk as the hen ages. Poult. Sci. 54, 1720–1723.

Rose, D., Gridgeman, N. T., & Fletcher, D. A. 1966. Solids content of eggs. Poult. Sci. 45, 221–226.

Rossi, M., & Pompei, C. 1995. Changes in some egg components and analytical values due to hen age. Poult. Sci.

Saki, A. A., Aliarabi, H., Hosseini Siyar, S. A., Salari, J., & Hashemi, M. 2014. Effect of a phytogenic

feed additive on performance, ovarian morphology, serum lipid parameters and egg sensory quality in laying hen. Vet. Res. forum an Int. Q. J. 5, 287–93.

Salajegheh, M. H., Ghazi, S., Mahdavi, R., & Mozafari, O. 2012. Effects of different levels of dried tomato pomace on performance, egg quality and serum metabolites of laying hens. African J. Biotechnol. 11, 15373–15379.

Senbeta, E., Zeleke, N., & Molla, Y. 2015. Attitudes and perceptions of consumers to chicken egg attributes in eastern ethiopia. J. Anim. Prod. Adv. 5, 705.

Sifri, M. 1995. Nutrient Requirements of Poultry.

Silversides, F. G. 1994. The haugh unit correction for egg weight is not adequate for comparing eggs from chickens of different lines and ages. J. Appl. Poult. Res. 3, 120–126.

Silversides, F. G., & Scott, T. A. 2001. Effect of storage and layer age on quality of eggs from two lines of hens. Poult. Sci. 80, 1240–1245.

Singh, R., Cheng, K. M., & Silversides, F. G. 2009. Production performance and egg quality of four strains of laying hens kept in conventional cages and floor pens. Poult. Sci. 88, 256–264.

Stadelman, W. J. 1986. Quality identification of shell eggs. Egg science and technology, 37–61.

Stadelman, W. J. 1992. World's poultry science association invited lecture. The Incredibly Functional Egg 1. Poult. Sci., 807–811.

Stadelman, W. J., & Cotterill, O. J. 1977. Egg science and technology.

Suk, Y. O., & Park, C. 2001. Effect of breed and age of hens on the yolk to albumen ratio in two different genetic stocks. Poult. Sci. 80, 855–858.

Surai, P. F., Fisinin, V. I., & Karadas, F. 2016. Antioxidant systems in chick embryo development. Part 1. Vitamin E, carotenoids and selenium. Anim. Nutr. 2, 1–11.

Surai, P. F., McDevitt, R. M., Speake, B. K., & Sparks, N. H. C. 1999. Carotenoid distribution in tissues of the laying hen depending on their dietary supplementation. WProc. Nutr. Soc. 5830A.

Surai, P. F., Speake, B. K., & Sparks, N. H. C. 2001. Carotenoids in avian nutrition and embryonic development. 2.antioxidant properties and discrimination in embryonic tissues. J. Poult. Sci. 38, 1–27.

Ternes, W., Acker, L., & Scholtyssek, S. 1944. Ei und eiprodukte. Poult. Sci.

Toussant, M. J., & Latshaw, J. D. 1999. Ovomucin content and composition in chicken eggs with different interior quality. J. Sci. Food Agric. 79, 1666–1670.

Travel, A., Nys, Y., & Bain, M. 2011. Effect of hen age, moult, laying environment and egg storage on egg quality.in improving the safety and quality of eggs and egg products: egg chemistry, production and consumption.

Tůmová, E., Gous, R. M., & Tyler, N. 2014. Effect of hen age, environmental temperature, and oviposition time on egg shell quality and egg shell and serum mineral contents in laying and broiler

breeder hens. Czech J. Anim. Sci. 59, 435–443.

USDA. 2000. Agricultural marketing service.- USDA egg-grading manual. agricultural handbook, Washington. Agric. Handb., 50.

Vuilleumier, J. P. 1969. The ‘Roche yolk colour fan‘—An instrument for measuring yolk colour. Poult. Sci. 48, 767–779.

Washburn, K. W. 1979. Genetic variation in the chemical composition of the egg. Poult. Sci. 58, 529–535.

Whitehead, C. C., Bowman, A. S., & Griffin, H. D. 1991. The effects of dietary fat and bird age on the weights of eggs and egg components in the laying hen. Br. Poult. Sci. 32, 565–574.

Williams, K. C. 1992. Some factors affecting albumen quality with particular reference to Haugh unit score. Worlds. Poult. Sci. J. 48, 5–16.

Yahav, S., Shinder, D., Razpakovski, V., Rusal, M., & Bar, A. 2000. Lack of response of laying hens to relative humidity at high ambient temperature. Br. Poult. Sci. 41, 660–663.

Youssef, A. W., Hassan, H. M. A., Ali, H. M., & Mohamed, M. A. 2013. Effect of probiotics, prebiotics and organic acids on layer performance and egg quality. Asian J. Poult. Sci., 1–10.

Zhao, J., Lin, H., Chen, Q., Huang, X., Sun, Z., & Zhou, F. 2010. Identification of egg’s freshness using NIR and support vector data description. J. Food Eng. 98, 408–414.

Zita, L., Ledvinka, Z., & Klesalová, L. 2013. The effect of the age of Japanese quails on certain egg quality traits and their relationships. Vet. Arh. 83, 223–232.

Zita, L., Tůmová, E., & Štolc, L. 2009. Effects of genotype, age and their interaction on egg quality in brown-egg laying hens. Acta Vet. Brno 78, 85–91.

Chapter 4

Influence of hen age and increased storage time on egg quality

Abstract

This trial investigated the effect of storage duration and its interaction with hen age on certain internal and external egg quality parameters. For this trial 480 Amberlink hen eggs were collected from eight different age groups (eight treatments) ranging from 21 to 77 weeks. Each group consisted out of 60 eggs. Each age group was divided into six groups of ten eggs, representing six different storage intervals (0, 15, 30, 45, 60 and 90 days). Storage intervals were used to investigate the effect of storage duration on egg quality parameters. Eggs were stored in a room where temperature was kept at 15.5˚C ± 2.2˚C and humidity at 75.8% ± 3.2% for the duration of the trial. Eggs were subjected to physical quality analysis to assess several egg quality parameters. Storage time had a significant effect on egg weight, shell weight, shell thickness, albumen weight, yolk weight, yolk colour a* and vitelline membrane integrity. Storage time had no significant effect on egg height, egg diameter, shell bumps, blood spots and meat spots. A significant interaction (hen age × storage time) for yolk height, Haugh unit, thin albumen height, thick albumen height, thin albumen spread, thick albumen spread, yolk colour L*, yolk colour b* and Roche colour fan was recorded. There was no significant interaction effect on any of the proximate constituents, which included moisture, protein, lipid and ash content. However, storage time, had a significant effect on the moisture and protein content. Noteworthy differences in egg quality were already observed within the first 15 days of storage. It can be concluded that most of the egg quality parameters were influenced either by storage time or by a combination between hen age and storage time, demonstrating the importance of these two factors when considering egg quality and egg safety.

Keywords: *albumen viscosity, carotenoids, Haugh unit, lysozyme-ovomucin complex, Roche colour fan, vitelline membrane, yolk colour*

4.1 Introduction

Several food products can be affected by shelf life, with shelf life depending on the type of food, storage duration and storage conditions. In comparison with other animal food products, eggs are one of the few food products of animal origin that can be stored for longer periods of time, because of their ability to function as a storage entity (Grashorn, 2016). However, eggs can rapidly decline in quality and easily spoil if optimal storage conditions are not met. These storage conditions include humidity, temperature and gaseous environment (Akyurek & Okur, 2009). These factors can be controlled (e.g. aircons and humidifiers) to maintain good egg quality, but only to a certain extent. With prolonged

storage, eggs undergo changes in physical (e.g. egg weight and albumen height) and proximate (e.g. protein and moisture) characteristics, which contribute to the final egg quality (Silversides & Scott, 2001).

During storage, the external and internal egg quality parameters can be altered. The most influential effect of storage time on egg quality is the loss of moisture and carbon dioxide (CO_2) from the internal component of the egg to the surrounding environment (Walsh *et al.*, 1995; Akyurek & Okur, 2009) leading to an increase in the albumen pH (Decuypere *et al.*, 2001). This increase in albumen pH and loss in moisture have detrimental effects on an array of quality characteristics. The loss of moisture leads to a direct decrease in total egg weight, proven by Stadelman (1986). However, increased storage time does not influence the shape (egg height and diameter) of the egg (Tilki & Saatci, 2004; Raji *et al.*, 2009). Shell thickness and shell weight can be influenced displaying a decrease in both parameters with prolonged storage (Kato *et al.*, 1970; Samli *et al.*, 2005). However, some studies indicated that there was no significant effect on these two parameters (Monira *et al.*, 2003; Curtis *et al.*, 2005).

As mentioned before, prolonged storage leads to a loss in moisture and increased albumen pH, influencing the internal quality the most. The most common measurement to assess internal egg quality, which has been used over the years is the Haugh unit (HU) (Williams, 1992; Robert, 2004). The HU is a logarithm of the height of the thick albumen adjusted to the total egg weight (Haugh, 1937). Higher HU values indicate better quality eggs and *vice versa* (Adamiec *et al.*, 2018). Storage can have an effect on total egg weight, as stated before and can also have an effect on the thick albumen height (Benton & Brake, 1996; Silversides & Scott, 2001), suggesting that the HU can change as a result of significant changes in total egg weight, thick albumen height or both with extended storage duration. While albumen weight decreases with an increase in storage time, yolk weight increases (Jin *et al.*, 2011; Akter *et al.*, 2014). This increase in yolk weight is due to the loss of structural integrity of the vitelline membrane (Figure 2.2) surrounding the egg yolk (Messens *et al.*, 2005). Decreased vitelline membrane strength increases the permeability of the membrane resulting in easier movement of moisture from the albumen to the yolk, giving rise to increased yolk weight and decreased yolk height (Kirunda & McKee, 2000; Jones, 2007). Barbosa *et al.* (2011) and Jin *et al.* (2011) showed that extended storage can also lead to a change in the yolk colour, due to structural disintegration of carotenoids, responsible for pigmentation (Britton & Khachik, 2009).

In addition to the change in the moisture content, increased storage time can have a substantial effect on the proximate composition of eggs. Studies done to investigate protein content changes during storage have been limited over the past years. There is a lot of controversy surrounding changes in egg protein content during storage. Some studies hypothesized that as storage time increased, the protein content, determined on a dry matter basis, will decrease due to change in the lysozyme-ovomucin complex (Yamamoto & Gutierrez, 1997). Others believe that the decrease is due to an increase in pH, resulting in an increase in the proteolysis activity (Omana *et al.*, 2011). On the other hand, Yetim and

Ockerman (1995) concluded that storage time had no effect on the total protein content. Ahn *et al.* (1997) concluded that the protein content and lipid content increased with an increase in storage time, however the protein and lipid content was determined on a wet basis. The increase in the protein and lipid content, reported by Ahn *et al.* (1997) was thus due to a decrease in moisture content. The total lipid content has also been shown to be altered during storage. A study done by Nascimento *et al.* (2008) showed that eggs stored for 60 days had a lower lipid content when compared to shorter storage times (0 and 30 days). They concluded that the decrease can be attributed to lipid oxidation, responsible for degradation of important lipid components such as polyunsaturated fatty acids, which can lower the risk of chronic heart diseases in humans (Lewis *et al.*, 2000; McClements & Decker, 2000). The lipid content thus contributes to the quality of the eggs as a higher polyunsaturated fatty acid content is considered healthier and thus affects consumers' acceptability (Song *et al.*, 2000). The proximate composition is thus of great importance for assessing egg quality as it affects consumers' acceptability (Song *et al.*, 2000) and the preservation thereof ensures eggs that are safe for consumption (Silversides & Scott, 2001).

As seen in Chapter 3, hen age can have a significant effect on most of the internal, external and proximate quality parameters of eggs. Numerous studies over the years have also shown that storage time can have an equally, if not greater influence on egg quality characteristics. However, research done on the interaction of storage time and hen age is limited and not fully understood. The results obtained in this research trial will provide further insight into egg quality when analysed during hyperspectral imaging discussed later in Chapter 5. Therefore, the aim of this study was to examine the effect of storage time and hen age on egg quality parameters, as well as the proximate composition of eggs.

4.2 Materials and Methods

4.2.1 Experimental design

For the purpose of this trial 480 Amberlink hen eggs were collected from Rosendal Poultry Farm situated 34 km from Stellenbosch University near Paarl (-33.738734, 19.029038). Information such as diet composition, feed intake, average daily gain, housing conditions and lighting program was not disclosed by the farm due to security purposes and will therefore not be discussed in the current study. However, hens received the same diet irrespective of age. The eggs were collected on the day they were laid. Eight groups of 60 eggs per group were collected from the farm and transported to Stellenbosch University where the eggs were subjected to near infrared hyperspectral imaging at the Department of Food Sciences (-33.925242, 18.871121) discussed in Chapter 5, followed by egg quality measurements at the Department of Animal Sciences (-33.931567, 18.867191). No cracked eggs were collected. The only difference between the eight egg groups (eight treatments) were the age of the hen, used to assess the influence of hen age on egg quality discussed in Chapter 3. Each hen age treatment

consisted of 60 eggs, which were divided into six storage interval groups consisting of 10 eggs. The first ten eggs were randomly selected for immediate analysis, on the day of collection (day 0). The remaining eggs were stored and randomly allocated to five different storage intervals which included 15, 30, 45, 60 and 90 days, respectively. These storage intervals was selected based on previous research (Williams & Pieterse, 2019). A total of 80 eggs were analysed at each storage interval. Eggs were stored at the Department of Animal Science, Stellenbosch University (-33.931567, 18.867191) in a dark room in reinforced egg trays, where temperature was kept at 15.5˚C ± 2.2˚C and humidity at 75.8% ± 3.2% for the duration of the trial.

4.2.2 Experimental procedure and data collection

Experimental procedure and data collection were carried out as explained in Section 3.2.2 (Chapter 3). All the egg quality parameters investigated are displayed in Table 3.1.

4.2.3 Statistical analysis

For statistical analysis the data analysis software system, STATISTICA, Version 9 (TIBICO Software Inc., 2018) was used. A one-way analysis of variances (ANOVA) was used to analyse the two effects (age and storage time), as well as their interaction on the different variables (egg weight, egg height, etc.). The Bonferroni least significant difference (LSD) *post hoc* test was used to see if there were any differences between the means of different storage intervals in terms of the variables. The same was done for the different hen ages. A significant level of 5% ($P \leq 0.05$) was accepted as being statistically significant. The incidence of banded eggshells, mottling and double yolk eggs were too low to allow for statistical analysis. When internal egg contents were investigated it was noted that all blood spot incidences occurred on both the yolk and albumen. The same was observed for meat spots. For this reason, yolk and albumen blood spots were combined and subjected to statistical analysis. The same was done for yolk and albumen meat spots. If a significant interaction (storage time × hen age) was observed, the main effects were not discussed. Significant hen age effects on egg quality parameters were not discussed, as it was already discussed in Chapter 3. The rate of change (slope) was calculated for each egg quality parameter that was significantly influenced by the interaction of storage time and hen age by plotting the line of best fit. The rate of change was used to test if egg quality parameters, that were influenced by the interaction of the main effects, would increase or decrease at the same rate between different aged hens as storage time progressed. The line of best fit could not be implemented on yolk colour L*, yolk colour b* and the Roche yolk colour fan, therefore the determination of the rate of change for these three parameters was not possible.

4.3 Results and Discussion

4.3.1 Effect of storage time on egg quality parameters

Table 4.1 display egg quality parameters that did not have a significant interaction (P>0.05) between storage time and hen age. However, some of these parameters were significantly influenced (P≤0.05) by the main effect, storage time. These parameters include egg weight, shell weight, shell thickness, albumen weight, yolk weight, yolk colour a* and vitelline membrane integrity. Storage time had no significant effect on egg height, egg diameter, shell bumps, blood spots and meat spots.

Table 4.1 The means ± standard deviations of egg quality parameters for eggs stored at different time intervals

Parameter		Egg Weight (g)	Egg Height (mm)	Egg Diameter (mm)	Shell Weight (g)
Treatment (Storage Time in Days)	0	59.47^{a} ± 5.93	54.79 ± 2.39	43.91 ± 1.65	8.05^{a} ± 0.73
	15	57.86^{b} ± 5.38	55.02 ± 2.39	43.50 ± 1.58	7.51^{b} ± 0.78
	30	57.44^{bc} ± 5.96	54.51 ± 2.24	43.76 ± 2.02	7.49^{bc} ± 0.79
	45	57.15^{c} ± 6.13	54.52 ± 2.58	43.61 ± 2.08	7.38^{bc} ± 0.84
	60	56.12^{d} ± 5.31	54.70 ± 2.48	43.94 ± 1.92	7.33^{bc} ± 0.72
	90	54.72^{e} ± 5.49	54.22 ± 2.37	44.19 ± 1.46	7.32^{c} ± 0.83
	P value	<0.01	0.16	0.11	<0.01
Parameter		Shell Thickness (mm)	Albumen Weight (g)	Yolk Weight (g)	Yolk Colour a*
Treatment (Storage Time in Days)	0	0.43^{a} ± 0.04	36.30^{a} ± 3.30	15.39^{e} ± 1.92	13.82^{b} ± 1.27
	15	0.43^{a} ± 0.03	34.55^{bc} ± 3.58	15.87^{cd} ± 1.85	12.84^{c} ± 1.13
	30	0.41^{b} ± 0.03	34.43^{bc} ± 3.82	15.64^{d} ± 1.82	14.32^{b} ± 0.92
	45	0.41^{bc} ± 0.03	35.54^{ab} ± 4.06	15.94^{c} ± 1.89	15.91^{a} ± 1.14
	60	0.40^{cd} ± 0.04	33.36^{c} ± 4.03	16.36^{b} ± 1.99	16.31^{a} ± 1.30
	90	0.39^{d} ± 0.03	29.33^{d} ± 4.58	16.65^{a} ± 2.38	16.35^{a} ± 1.14
	P value	<0.01	<0.01	<0.01	<0.01

($^{a, b, c, d, e}$) Means with different superscripts within the same column differ significantly (P≤0.05).

It is noted in Table 4.1 and Figure 4.1 that storage time had a significant effect on egg weight (P<0.01). The egg weight includes the weight of the shell as well as the inner content. Prolonged storage time led to an overall decrease in total egg weight. Eggs were at their heaviest on the day they were laid (day 0) and showed the lowest weight, in grams at the end of the trial (day 90). From Figure 4.1, it is clear that the biggest reduction in egg weight occurred within the first 15 days of storage. This result coincide with the study done by Gavril and Usturoi (2012), concluding that the biggest reduction in egg weight was within the first 14 days of storage, which was also confirmed later by Eke *et al.* (2013). This decrease in egg weight, due to prolonged storage can be ascribed to the reduction in the weight of the albumen, as these two parameters are directly correlated to each other (Stadelman, 1986b; Kul & Seker,

2004). This statement was confirmed when observing Table 4.1, which indicates that there was a significant reduction in the albumen weight as well and a strong correlation coefficient of 0.90 between egg weight and albumen weight. These results are comparable with numerous other studies done over the years (Hill & Hall, 1980; Reis *et al*., 1997; Bhale *et al*., 2003; Samli *et al*., 2005; Pamarin *et al*., 2009; Raji *et al*., 2009; Khan *et al*., 2014; Grashorn, 2016) all concluding that increased storage time led to a reduction in total egg weight due to decrease in albumen weight.

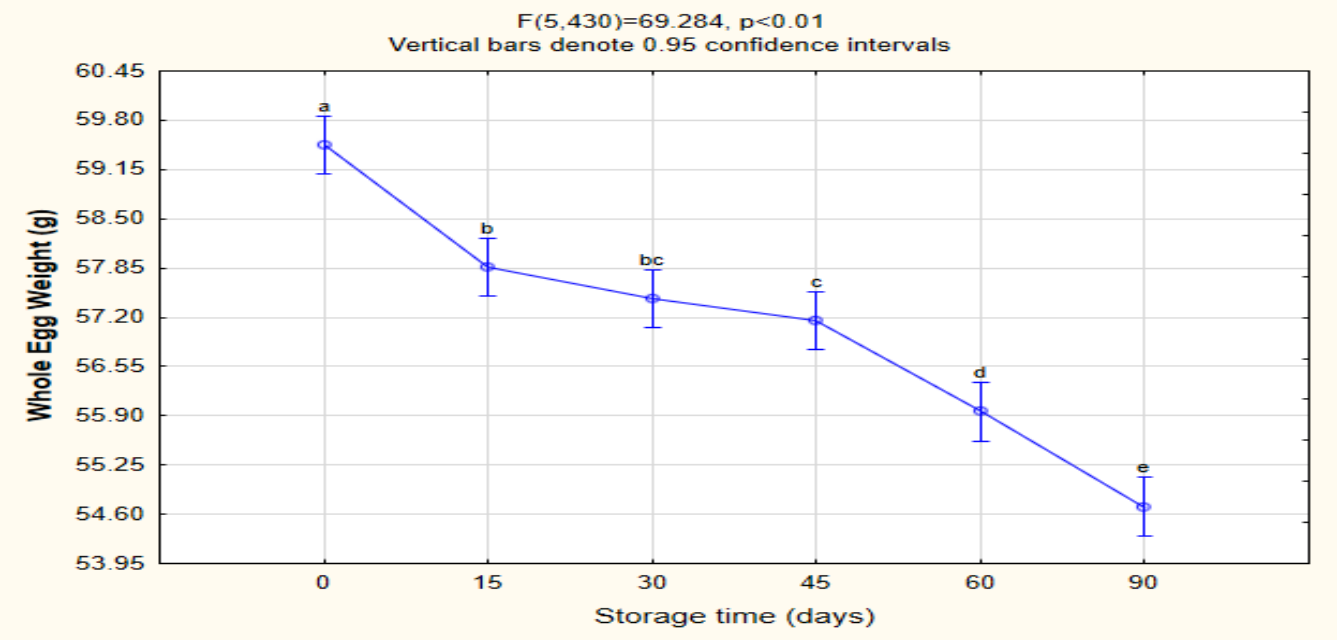


Figure 4.1 Graph showing the change in the mean whole egg weight (g) produced by Amberlink hens stored for different time intervals ranging from 0 to 90 days.

Significant differences were observed for shell weight (P<0.01) and shell thickness (P<0.01) with increased storage time (Table 4.1). The decrease in shell weight in the current study coincides with results obtained by Kato *et al.* (1970), Samli *et al.* (2005) and Jin *et al.* (2011), also reporting a decrease, as storage time progressed, but they concluded that the cause for the change in shell weight over time is unknown. The decrease in shell thickness was not noted in other storage trials cited (Tilki & Saatci, 2004; Callejo Ramos *et al.*, 2010). Grashorn (2016) on the other hand found similar results to that of the current study and proposed that the decrease in shell weight and shell thickness, with increased storage time, might be attributed to shrinkage and deterioration of the cuticle, inner shell membrane or both. The membrane's shrinkage might be due to aeration, which occurs during storage when moisture between the inner and outer shell membrane is displaced by gas (mostly oxygen and carbon dioxide) to the storage environment, resulting in drying of the shell membranes and thus shrinkage as well as possible deterioration (Seymour & Piiper, 1988; Grashorn, 2016).

Storage time significantly influence albumen weight (P<0.01) and yolk weight (P<0.01) (Table 4.1). Albumen weight is one of the egg quality parameters that is influenced the most by prolonged storage

(Williams, 1992; Robert, 2004). As the storage time progressed, the albumen weight decreased from 36.30 g at day 0 to 29.33 g at day 90 (Table 4.1). During storage there is an increased loss of carbon dioxide through the eggshell pores resulting in an increase in albumen pH. The increase in pH is due to the conversion of the remaining carbon dioxide to carbonate. The albumen pH of a freshly laid egg is relatively neutral with an initial pH of 7.6, it can reach up to 9.2 or higher, after 14 days of storage (Silversides & Budgell, 2004). The higher pH disrupts the hydrogen bonds between the side chains in the vitelline membrane, changing the osmosis equilibrium between the yolk and albumen (Dawes, 1975). Changes in the osmotic equilibrium facilitates the movement of moisture from the albumen to the yolk (Brake *et al.*, 1997; Kirunda & McKee, 2000). This would result in an increase in yolk weight and a decrease in albumen weight, which was confirmed in the current study (Table 4.1). This was also confirmed by a correlation analysis between albumen weight and yolk weight, showing a negative correlation (r = -0.66) between these two parameters. Albumen moisture is not only lost to the yolk, but to the storage environment as well. The albumen is enclosed by the eggshell which contain a vast amount of shell pores. The shell is covered by a layer which consists of glycoproteins, lipids, polysaccharides and hydroxyapatite crystals known as the cuticle (Wellman-Labadie *et al.*, 2008). The cuticle forms a block arrangement in the shell pores, which facilitates exchange of gases and prevents moisture loss (Grashorn, 2016). However, during increased storage periods the cuticle proteins become less glycosylated (decreased structural stability), which results in the weakening of the cuticle layer (Rodríguez-Navarro *et al.*, 2013). This leads to higher moisture loss through the shell pores, further explaining the decrease noticed in albumen weight in the current study. It is shown in Table 4.10 that the moisture content decreased, as storage time progressed and that there was a positive correlation (r = 0.67) between the albumen weight and moisture content. The results coincides with various studies that found similar results in terms of albumen weight and yolk weight, over the last 20 years (Altan *et al.*, 1997; Lapão *et al*., 1999; Silversides & Scott, 2001; Tilki & Saatci, 2004; Samli *et al*., 2005; Akyurek & Okur, 2009; Demirel & Kirikçi, 2009; Jin *et al.*, 2011; Eke *et al*., 2013; Grashorn, 2016).

Increased storage time led to a significant ($P<0.01$) increase in the yolk colour a* or redness (Table 4.1), ranging from 13.82 to 16.35, suggesting that as the storage time increased the yolk colour of the stored eggs had a slightly redder appearance. In contrast, Bhale *et al.* (2003) reported a decrease in the yolk redness as storage time increased. It is however, unclear why Bhale *et al.* (2003) reported a decrease in yolk redness. An increase in yolk redness can usually be ascribed to increase in the level of carotenoids (xanthophyll) in the diets of layers (Beardsworth & Hernandez, 2004) however, the eggs used in the current trial were all laid by hens receiving the same diet, therefore this was not a viable explanation for the increase in yolk redness. Alternatively this increased yolk redness (colour a*) may have been the result of changes in the carotenoid concentration in the yolk (Robert, 2004). Carotenoids contain a large number of double bonds in their molecules, which can be altered through oxidation, and

this is accelerated by increased storage time (Barbosa *et al.*, 2011). The oxidation of carotenoids (xanthophylls) leads to yolks with darker colour as xanthophylls contribute mostly to a light yellow or orange colour in egg yolks (Surai *et al.*, 2001). It is thus possible that the increase in redness is due to a decrease in carotenoid concentration resulting in darker yolks, which could increase the a* measurements taken by the spectrophotometer, which is associated with a darker red colour. However, this assumption cannot be confirmed as the carotenoid concentration of the samples was not measured in the current study.

For the categorical data, storage time had a significant effect on the vitelline membrane integrity ($P = 0.01$) but had no significant effect on shell bumps, blood spots and meat spots ($P>0.05$). The vitelline membrane integrity can be compromised by two contributing factors. Firstly, as mentioned earlier, increased storage time can lead to an increase in pH due to a conversion of carbon dioxide to carbonate. Carbonate disrupts the hydrogen bonds between the side chains in the vitelline membrane, which are responsible for the structural stability. Secondly, the yolk also increased in size due to movement of moisture from the albumen to the yolk, resulting in increased stretching of the vitelline membrane. The loss of structural integrity and increased vitelline membrane stretching could contribute to a higher occurrence of rupturing yolks. It was expected that increased storage time would lead to an increased incidence of yolks rupturing (compromised vitelline membrane) as the vitelline membrane protects the inner yolk fluid (McNally, 1943; Kirunda & McKee, 2000; Jones & Musgrove, 2005). This assumption was confirmed in the current study as increased storage time led to a significant ($P = 0.01$) increase in the occurrence of the vitelline membrane integrity being compromised as most of the ruptures occurred at 90 days storage ($n = 28$) followed by 60 days ($n = 20$), 45 days ($n = 10$), 30 days ($n = 6$) and no ruptures at 15 and 0 days of storage ($n = 0$). Therefore, prolonged storage increased the occurrence of ruptured egg yolks. Numerous studies that assessed vitelline membrane integrity as a factor of storage time confirmed this result (Burley & Vadehra, 1989; Benton & Brake, 1996; Kirunda & McKee, 2000; Kaspers, 2016).

4.3.2 Influence of storage time and hen age on egg quality

For this section focus was placed on the interaction between storage time and hen age on egg quality parameters. Significant interactions ($P<0.05$) were observed for yolk height, HU, thin albumen height, thick albumen height, thin albumen spread, thick albumen spread, yolk colour L*, yolk colour b* and Roche colour fan values.

Yolk height was significantly affected ($P<0.01$) by the interaction between hen age and storage time (Table 4.2). Table 4.2 shows a clear indication that there was an overall decrease in yolk height as storage time progressed and in Chapter 3 it was concluded that there was an increase in yolk height as hen age increased. The decrease in yolk height can be attributed to increased stretching of the vitelline

membrane (Brake *et al.*, 1997). As storage time increases the vitelline membrane structure is compromised due to increase in albumen pH, which is caused by the conversion of carbon dioxide to carbonate. Carbonate disrupts the hydrogen bonds between the side chains of the vitelline membrane and alters the osmosis equilibrium between the albumen and yolk. This facilitates the movement of moisture from the albumen to the yolk, increasing the yolk size and thus increasing the vitelline membrane elasticity (Burley & Vadehra, 1989; Jacob *et al.*, 2000). Increased elasticity contributes to an increase in yolk diameter and a decrease in yolk height because the yolk weight increases with prolonged storage as discussed above. From Table 4.2 it is observed that the rate of change for most of the older hens was higher compared to the younger hens. It can thus be concluded that as the storage time increased the yolk height declined, but there was a greater decline in eggs collected from older hens than younger ones. These results coincide with Akyurek and Okur (2009) who also found a significant interaction between the main effects, hen age and storage time on yolk height, concluding bigger change in yolk height of eggs laid by older hens compared to younger ones with prolonged storage. From Table 4.2 it appears that there was already a substantial reduction in yolk height within the first 15 days of storage as well as between 60 and 90 days of storage. This result was consistent with that of Caner, (2005) also reporting a considerable reduction in the yolk height within the first 14 days of storage. The noteworthy reduction in yolk height (Table 4.2) between 60 and 90 days of storage is likely due to a longer storage interval (30 days).

The HU is directly related to the thick albumen height and it was thus expected that HU would also show a significant interaction between storage time and the age of the hen. This assumption was confirmed when observing Table 4.3 and Figure 4.2 displaying a significant interaction ($P<0.01$) between the main factors. As mentioned before, HU is directly related to the thick albumen height (Haugh, 1937). Thus, a decrease in thick albumen height will lead to a decrease in the HU of eggs. The decrease in thick albumen height (Table 4.5) with increased storage is the main contributing factor that led to this decrease (Williams, 1992; Tona *et al.*, 2004). As discussed in Chapter 3, increase in hen age can decrease HU and Williams (1992) confirmed that increased storage time can also decrease HU. It was expected that eggs laid by older hens would show a more substantial decline in HU as storage time increased compared to eggs laid by younger hens. This was confirmed by the results presented in Table 4.3 and as shown in Figure 4.2. The rate of change in HU showed greater decrease in eggs laid by older hen ages compared to eggs laid by younger hens as storage time increased. The lowest rate of change was at 21 weeks (-0.007 HU per storage interval) and the greatest change was at week 77 (-0.054 HU per storage interval). This is in accordance with the results shown in Figure 4.2, where eggs laid by older hen ages (e.g. 69 and 77 weeks) had steeper slopes compared to eggs laid by younger hen ages (e.g. 21 and 29 weeks) as storage time progressed. It can thus be concluded that eggs laid by older hens showed a more substantial decrease in HU as time progressed when compared to eggs.

Table 4.2 The means and standard deviations of yolk height (mm) for eggs stored at different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)						[1]ROC
	0	15	30	45	60	90	
21	18.51^{d} ± 0.59	16.72^{ij} ± 0.72	15.46^{mno} ± 0.56	14.92^{pq} ± 0.57	14.33^{rs} ± 0.52	12.14^{v} ± 0.58	-0.007
29	19.48^{c} ± 0.63	17.88^{ef} ± 0.65	17.51^{fgh} ± 0.65	15.95^{klm} ± 0.75	15.36^{nop} ± 0.54	13.38^{t} ± 0.59	-0.004
37	19.75^{bc} ± 0.43	17.09^{ef} ± 0.52	17.16^{hi} ± 0.50	15.72^{lmn} ± 0.64	14.93^{opqr} ± 0.34	13.25^{t} ± 0.74	-0.009
45	19.68^{c} ± 0.53	17.24^{h} ± 0.64	16.20^{kl} ± 0.55	15.30^{nop} ± 0.69	14.51^{qrs} ± 0.65	12.56^{uv} ± 0.67	-0.013
53	19.88^{bc} ± 0.39	17.76^{efg} ± 0.47	17.28^{gh} ± 0.48	15.80^{klmn} ± 0.61	14.62^{qrs} ± 0.49	12.03^{v} ± 0.54	-0.005
61	19.73^{bc} ± 0.52	18.08^{de} ± 0.50	16.15^{kl} ± 0.40	15.02^{opq} ± 0.63	14.22^{s} ± 0.72	12.93^{tu} ± 0.63	-0.016
69	20.21^{ab} ± 0.49	17.76^{efg} ± 0.60	16.34^{jk} ± 0.30	15.77^{lmn} ± 0.53	14.87^{pqr} ± 0.68	13.43^{t} ± 0.48	-0.024
77	20.42^{a} ± 0.38	17.77^{efg} ± 0.62	16.36^{jk} ± 0.60	15.37^{nop} ± 0.42	15.06^{opq} ± 0.78	12.81^{tu} ± 0.72	-0.017
Source of variance							*
Storage time	*<0.01*						
Hen age	*<0.01*						
Storage time × Hen age	*<0.01*						

[1]ROC = Rate of change; * = ROC differed significantly between hen ages.

Table 4.3 The means and standard deviations of the Haugh unit for eggs stored at different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)						[1]ROC
	0	15	30	45	60	90	
21	101.37^{a} ± 1.85	80.76^{e} ± 1.84	75.50^{f} ± 1.82	65.94^{h} ± 1.98	57.90^{j} ± 1.9	55.13^{k} ± 1.51	-0.007
29	92.48^{b} ± 1.36	72.80^{g} ± 1.61	58.03^{j} ± 1.64	43.01^{o} ± 1.74	35.17^{s} ± 1.85	25.21^{uv} ± 1.83	-0.015
37	92.17^{b} ± 1.87	62.01^{i} ± 1.65	50.71^{m} ± 1.74	34.71^{s} ± 1.73	32.47^{t} ± 1.75	24.19^{v} ± 1.85	-0.032
45	89.82^{c} ± 1.48	53.05^{l} ± 1.55	45.79^{n} ± 1.84	32.43^{t} ± 1.69	26.37^{u} ± 1.58	19.40^{A} ± 2.17	-0.030
53	89.26^{c} ± 1.21	61.58^{i} ± 1.58	38.24^{q} ± 2.03	23.70^{vw} ± 1.75	21.72^{wxy} ± 1.61	17.67^{B} ± 1.52	-0.036
61	89.39^{c} ± 1.44	62.38^{i} ± 1.39	40.64^{p} ± 1.63	22.20^{wx} ± 1.04	19.08^{A} ± 1.41	16.07^{C} ± 1.60	-0.037
69	87.02^{d} ± 1.39	62.67^{i} ± 1.80	36.22^{rs} ± 1.85	21.33^{xy} ± 1.38	17.09^{B} ± 1.91	15.02^{D} ± 2.24	-0.034
77	86.01^{d} ± 1.14	49.31^{m} ± 1.77	37.63^{qr} ± 1.43	20.14^{yz} ± 1.97	19.16^{A} ± 2.00	13.83^{E} ± 2.03	-0.054
Source of variance							*
Storage time	*<0.01*						
Hen age	*<0.01*						
Storage time × Hen age	*<0.01*						

[1]ROC = Rate of change; * = ROC differed significantly between hen ages.

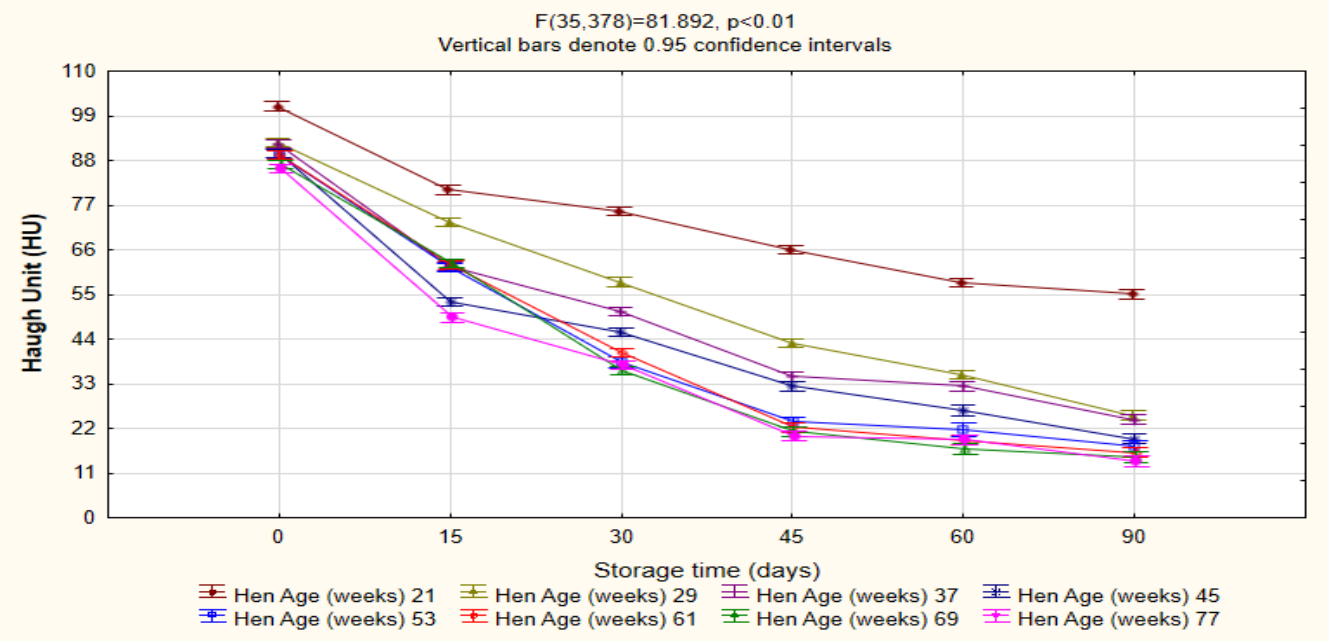


Figure 4.2 Graph illustrating the change in the Haugh unit (HU) of eggs stored for 90 days, laid by Amberlink hens with ages ranging from 21 to 77 weeks.

laid by younger hens. It can also be concluded that the biggest decrease in HU was in the first 15 days of the trial, illustrating a sharp decline compared to the rest of the storage intervals as shown in Figure 4.2, which is also reported in Table 4.3, coinciding with numerous studies (Bhale *et al.*, 2003; Dudusola, 2009; Kemps *et al.*, 2010).

Thin albumen height (Table 4.4) and thick albumen height (Table 4.5) were both affected significantly ($P<0.01$) by the interaction of storage time and hen age. From Table 4.4 and Table 4.5 it was noted that the biggest decrease in the thin and thick albumen height was between 0 and 15 days of storage, which was consistent with Jones and Musgrove (2005), reporting the biggest reduction in the first two weeks of storage. Both thick and thin albumen height deteriorated as storage time progressed, which can once again be ascribed to the increase in albumen pH due to carbon dioxide dissolving in egg moisture, which is converted to carbonate (Dawes, 1975; Silversides & Budgell, 2004). As mentioned in Chapter 3, albumen consists of various proteins, but the most important ones in terms of albumen height, is the stability of the lysozyme-ovomucin complex (Toussant & Latshaw, 1999). This complex gives the thick and thin albumen its gel like structure, giving rise to the thick and thin albumen layers. The lysozyme-ovomucin complex remains stable until the pH value of the albumen is equal to 9.2 (Lewko & Gornowicz, 2009), which can be reached within the first 14 days of storage (Silversides & Budgell, 2004). As storage time progresses, the pH continues to increase and the isoelectric point of the lysozyme complex is exceeded. This results in a complete dissociation of the disulphide bonds between lysozyme and α-ovomucin, which is covalently linked to form the complex (Hayakawa *et al.*, 1983; Yamamoto & Gutierrez, 1997). This leads to alteration in the thick and thin albumen stability,

Table 4.4 The means and standard deviations of thin albumen height (mm) for eggs stored at different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)						[1]ROC
	0	15	30	45	60	90	
21	2.85^{a} ± 0.22	2.56^{b} ± 0.15	2.21^{c} ± 0.27	1.91^{d} ± 0.15	1.85^{de} ± 0.19	1.72^{efg} ± 0.29	-0.012
29	2.20^{c} ± 0.12	1.72^{efg} ± 0.20	1.71^{efg} ± 0.29	1.58^{fghi} ± 0.11	1.43^{ijkl} ± 0.23	1.30^{klmno} ± 0.16	-0.008
37	2.14^{c} ± 0.22	1.58^{fghi} ± 0.15	1.48^{hijk} ± 0.27	1.40^{ijklm} ± 0.15	1.25^{lmnop} ± 0.22	1.24^{mnop} ± 0.15	-0.008
45	2.13^{c} ± 0.32	1.54^{ghi} ± 0.28	1.35^{jklmn} ± 0.23	1.24^{mnop} ± 0.11	1.26^{lmnop} ± 0.17	1.26^{mnop} ± 0.12	-0.008
53	1.89^{de} ± 0.32	1.65^{fgh} ± 0.22	1.27^{lmnop} ± 0.17	1.27^{lmnop} ± 0.20	1.14^{opqr} ± 0.12	1.16^{opqr} ± 0.09	-0.008
61	1.86^{de} ± 0.10	1.55^{ghi} ± 0.26	1.29^{lmno} ± 0.26	1.25^{lmnop} ± 0.19	1.08^{pqrs} ± 0.09	1.03^{qrs} ± 0.04	-0.008
69	1.76^{def} ± 0.11	1.49^{hij} ± 0.14	1.26^{lmnop} ± 0.22	1.25^{lmnop} ± 0.21	1.08^{pqrs} ± 0.15	1.00^{rs} ± 0.09	-0.008
77	1.64^{fgh} ± 0.17	1.41^{ijklm} ± 0.23	1.25^{lmnop} ± 0.31	1.21^{nopq} ± 0.33	1.01^{grs} ± 0.08	0.90^{s} ± 0.12	-0.008
Source of variance							
Storage time	*<0.01*						
Hen age	*<0.01*						
Storage time × Hen age	*<0.01*						

[1]ROC = Rate of change.

Table 4.5 The means and standard deviations of thick albumen height (mm) for eggs stored at different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)						[1]ROC
	0	15	30	45	60	90	
21	9.69^{a} ± 0.51	5.78^{e} ± 0.44	4.96^{g} ± 0.65	3.82^{i} ± 0.45	3.08^{jk} ± 0.33	3.20^{j} ± 0.57	-0.012
29	8.74^{b} ± 0.59	5.37^{f} ± 0.34	3.85^{i} ± 0.59	2.65^{lm} ± 0.50	2.17^{no} ± 0.46	1.58^{qrstuv} ± 0.40	-0.030
37	8.70^{b} ± 0.39	4.25^{h} ± 0.27	3.30^{j} ± 0.40	2.11^{op} ± 0.54	1.81^{opqr} ± 0.46	1.66^{qrstu} ± 0.45	-0.051
45	8.02^{c} ± 0.33	4.49^{h} ± 0.47	2.98^{jkl} ± 0.42	2.06^{op} ± 0.54	1.76^{pqrs} ± 0.44	1.33^{tuv} ± 0.33	-0.054
53	8.02^{c} ± 0.28	4.27^{h} ± 0.51	2.55^{mn} ± 0.47	1.72^{pqrst} ± 0.48	1.18^{v} ± 0.36	1.35^{tuv} ± 0.35	-0.048
61	8.03^{c} ± 0.35	4.37^{h} ± 0.50	2.65^{lm} ± 0.33	1.65^{qrstu} ± 0.42	1.53^{qrstuv} ± 0.40	1.60^{qrstuv} ± 0.37	-0.054
69	7.26^{d} ± 0.38	4.37^{h} ± 0.30	2.50^{mn} ± 0.50	1.74^{pqrs} ± 0.32	1.32^{stuv} ± 0.19	1.30^{uv} ± 0.24	-0.056
77	7.59^{d} ± 0.23	4.44^{h} ± 0.54	2.72^{klm} ± 0.55	1.75^{pqrs} ± 0.34	1.97^{opq} ± 0.33	1.43^{rstuv} ± 0.31	-0.084
Source of variance							*
Storage time	*<0.01*						
Hen age	*<0.01*						
Storage time × Hen age	*<0.01*						

[1]ROC = Rate of change; * = ROC differed significantly between hen ages.

resulting in the reduction in height and leads to liquefaction or albumen thinning (Omana *et al.*, 2011). As mentioned in section 3.3.2, increased hen age leads to a decrease in the thick and thin albumen height as well (Silversides & Scott, 2001; Roberts & Ball, 2005). It is thus anticipated that the albumen height would show a greater reduction for older hens than that of younger ones. This assumption was not confirmed for the thin albumen height as there was no significant differences in the rate of change between the hen ages (Table 4.4), but was confirmed for the thick albumen height (Table 4.5). In Table 4.4, the rate of change stays the same between the hen ages with increased storage time, with the exception of eggs laid by hens at 21 weeks of age (-0.012 mm per storage interval). Results in Table 4.4. suggest that thin albumen height declined at the same rate as storage time increased regardless of hen age. It is possible that eggs laid by all hen ages showed similar rates of change as storage time increased due to minor changes in the thin albumen height. This occurrence was also reported by Gennadios *et al.* (1998) stating that during increased storage, the thin albumen height reduction is substantially low and not as pronounced as the decrease in thick albumen height in eggs, laid by different aged hens. On the other hand, when the thick albumen height was observed in Table 4.5, the rate of change for the younger hens (21 weeks = -0.012 mm per storage interval; 29 weeks = -0.030 mm per storage interval) was smaller compared to that of the older hens (69 weeks = -0.056 mm per storage interval; 77 weeks = -0.084 mm per storage interval). This confirms that over time, there was a greater decrease in the thick albumen height in eggs laid by older hens compared to that of younger hens. It can also be concluded that there was a greater decrease in the thick albumen height compared to thin albumen height, since the thin albumen height showed a rate of change range between -0.008 mm and -0.012 mm (Table 4.4), whilst the rate of change for the thick albumen ranged between -0.012 mm and -0.084 mm per storage interval (Table 4.5). Gennadios *et al.* (1998) and Curtis *et al.* (2005) also reported a greater decrease in the thick albumen height compared to thin albumen height with increased storage.

Both thin and thick albumen spreading was significantly affected ($P<0.01$) by the interaction between storage time and hen age (Table 4.6 and Table 4.7, respectively). Some of the mean values in Table 4.6 and Table 4.7 displayed high standard deviations, which was due to the assignment of different categories to eggs belonging to the same age group (Figure 3.1, Chapter 3). For example, in Table 4.7, eggs collected from hens which were 53 weeks old and which have been stored for 0 days had a mean thick albumen spread value of 64 mm and a standard deviation of 29.51 mm. This was due to the fact that eight out of the ten eggs from this age and storage group, belonged to a category one spreading distance, whilst two eggs belonged to a category two spreading distance. It is also observed in Table 4.6 and Table 4.7 that high standard deviations occurred mainly at younger hen age groups. This suggested that there was higher biological variation between eggs of the same group laid by younger hen ages. This might be due to younger bird's reproductive system still adjusting to the laying

cycle (Jacob *et al.*, 2003). The thick and thin albumen spreading distance is negatively correlated with HU (Christensen *et al.*, 2001; Kemps *et al.*, 2010). In other words, a decrease in the HU value would correspond to an increase in the thin and thick albumen spreading distance from the egg yolk (Leeson & Caston, 1997). This statement was partially confirmed in the current study as there was a strong negative correlation between HU and thick albumen height (r = -0.82), whilst the correlation between HU and thin albumen height was not as distinct (r = -0.64). This result suggests that changes in the thin albumen spreading are not as pronounced as the thick albumen spreading distance between different hen ages as storage time progressed. As mentioned before, increased storage time led to the decrease in thick and thin albumen height. This was due to structural changes in the lysozyme-ovomucin complex resulting in the liquification or thinning of the albumen (Omana *et al.*, 2011). This again leads to a decrease in the viscosity of the thin and thick albumen (Pamarin *et al.*, 2009). The spreading distance of the thin and thick albumen is also related to the total egg weight, increasing with an increase in hen age, as reported in Chapter 3. It was already established that the HU is influenced by the interaction of the main effects and decreases with hen age and with increased storage time. Thus, it was expected that the thin and thick albumen spreading distance from the yolk would be greater in eggs laid by older hens and in eggs stored for longer periods. This statement was only confirmed for thick albumen spreading, reported in Table 4.7. The rate of change for the thin albumen spreading were similar between the hen ages and as storage time increased (Table 4.6). It can be concluded that the thin albumen spreading distance increased at the same rate between hen ages as storage time increased. This is once again a confirmation that the changes in the thin albumen are not as pronounced as changes in the thick albumen. The thick albumen spreading distance on the other hand showed differences in the rate of change between hen ages. Table 4.7 showed a greater rate of change in eggs laid by older hen ages (69 weeks = 0.046 mm per storage interval; 77 weeks = 0.040 mm per storage interval) compared to eggs laid by younger hens (21 weeks = 0.011 mm per storage interval; 29 weeks = 0.031 mm per storage interval). The thick albumen spreading showed a greater rate of change (Table 4.7) compared to the rate of change of the thin albumen spreading (Table 4.6). The thin albumen spreading distance ranged from 124 mm to 219 mm whilst the thick albumen spreading distance ranged from 50 mm to 213 mm. This suggests that as storage time increased, there was a greater increase in the spreading distance for the thick albumen compared to the thin albumen.

Table 4.6 The means and standard deviations of thin albumen spreading (mm) for eggs stored at different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)						[1]ROC
	0	15	30	45	60	90	
21	124^{n} ± 34.62	179^{hijkl} ± 8.43	173^{jkl} ± 20.55	177^{ijkl} ± 6.32	175^{jkl} ± 22.97	183^{ghijk} ± 10.32	0.010
29	159^{m} ± 34.54	181^{hijkl} ± 25.39	187^{fghij} ± 10.32	193^{defgh} ± 6.32	191^{efghi} ± 8.43	197^{cdefg} ± 7.07	0.010
37	175^{jkl} ± 21.66	183^{ghijk} ± 14.52	191^{defgh} ± 8.43	201^{bcdef} ± 13.49	203^{bcde} ± 10.69	205^{abcde} ± 14.14	0.011
45	168^{lm} ± 35.67	183^{ghijk} ± 23.81	193^{defgh} ± 6.32	200^{cdef} ± 9.25	202^{bcde} ± 10.35	207^{abcd} ± 10.32	0.010
53	172^{klm} ± 30.47	187^{fghij} ± 13.98	197^{cdef} ± 7.07	202^{bcde} ± 10.35	203^{bcde} ± 10.32	209^{abc} ± 9.66	0.016
61	179^{hijkl} ± 8.43	193^{defgh} ± 11.35	193^{defgh} ± 11.35	201^{bcdef} ± 9.66	207^{abcd} ± 14.88	209^{abc} ± 13.49	0.010
69	181^{hijkl} ± 9.66	193^{defgh} ± 14.75	197^{cdef} ± 11.35	203^{bcde} ± 10.32	207^{abcd} ± 13.98	215^{ab} ± 13.33	0.020
77	183^{ghijk} ± 10.32	197^{cdef} ± 14.75	201^{bcdef} ± 9.66	208^{abc} ± 10.00	209^{abc} ± 9.75	219^{a} ± 12.64	0.017
Source of variance							
Storage time	*<0.01*						
Hen age	*<0.01*						
Storage time × Hen age	*<0.01*						

[1]ROC = Rate of change.

Table 4.7 The means and standard deviations of thick albumen spreading (mm) for eggs stored for different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)						[1]ROC
	0	15	30	45	60	90	
21	50^{s} ± 0.00	92^{lmnop} ± 36.14	71^{pqrs} ± 33.81	97^{klmno} ± 44.17	112^{jklm} ± 23.33	135^{hij} ± 40.48	0.011
29	57^{rs} ± 22.13	57^{rs} ± 22.13	106^{klmn} ± 29.51	106^{klmn} ± 29.51	117^{ijkl} ± 41.84	197^{abc} ± 7.07	0.031
37	50^{s} ± 0.00	78^{opqr} ± 36.14	90^{mnop} ± 45.73	140^{ghi} ± 33.45	151^{fgh} ± 29.39	195^{abc} ± 0.00	0.032
45	50^{s} ± 0.00	78^{opqr} ± 36.14	92^{lmno} ± 36.14	166^{defg} ± 29.85	175^{cdef} ± 24.55	197^{abc} ± 11.35	0.036
53	64^{qrs} ± 29.51	78^{opqr} ± 36.14	116^{ijklm}± 47.41	161^{efg} ± 35.65	183^{bcde} ± 27.28	203^{ab} ± 10.32	0.041
61	57^{rs} ± 22.13	71^{pqrs} ± 33.81	113^{jklm} ± 22.13	185^{bcde} ± 35.65	195^{abc} ± 10.69	201^{abc} ± 16.46	0.040
69	64^{qrs} ± 29.51	83^{nopq} ± 46.07	120^{ijk} ± 0.00	193^{abcd} ± 14.75	191^{abcd} ± 28.09	211^{a} ± 15.77	0.046
77	71^{pqrs} ± 33.81	92^{lmnop} ± 36.14	135^{hij} ± 40.48	195^{abc} ± 14.14	200^{abc} ± 9.75	213^{a} ± 17.51	0.040
Source of variance							*
Storage time	*<0.01*						
Hen age	*<0.01*						
Storage time × Hen age	*<0.01*						

[1]ROC = Rate of change; * = ROC differed significantly between hen ages.

Yolk colour L* and yolk colour b* was significantly affected by the interaction between storage time and hen age, ($P<0.01$) as seen in Table 4.8 and Table 4.9, respectively. Carotenoids, more specifically xanthophylls, are the main compounds that determine colour in egg yolks (Britton & Khachik, 2009). In Table 4.8 it is reported that the yolk (colour L*) lightness increased for all hen ages within the first 15 days and decreased thereafter until day 90. The increase in yolk lightness in the first 15 days might be attributed to the accumulation of moisture by the yolk resulting in an increase in weight and size (Table 4.1). The increase in the moisture content of the yolk could have led to the dilution of the pigments, which are present in the yolks. Barreras Serrano *et al.* (2016) also concluded that there was an increase in yolk lightness after 6 days of storage, which was attributed to an increase in moisture content in the yolk with increased storage, diluting the pigments which are present. This would explain the increase in yolk lightness (L*) observed between days 0 and 15 (Table 4.8). The decrease in yolk lightness after 15 days might be attributed to a decrease in the carotenoid content due to oxidation (Nys, 2000). Britton (1995) explained that the stability of the carotenoids in food products can be affected by storage due to the presence of a polyene chain in the structure of carotenoids, that is highly susceptible to oxidation. Oxidation leads to a geometrical transformation in the double bonds of the polyene chain, resulting in an unstable carotenoid, which leads to loss in colour (Nys, 2000). During storage of eggs, carbon dioxide is not only lost to the environment, but eggs can also absorb oxygen through the shell pores (Tsai *et al.*, 2006). It is thus possible that the decrease in lightness from day 15 to 90 can be ascribed to the oxidation of carotenoids resulting in a change in the structural integrity and a loss in colour, which led to darker colour yolks as storage time progressed. Barbosa *et al.* (2011) found similar results with egg yolks displaying a significant reduction ($P<0.05$) in yolk pigmentation (lightness) after 28 days of storage, resulting in the yolk colour becoming slightly darker. In Section 3.3.2 it was concluded that eggs laid by older hens displayed yolks with higher L* values, suggesting an increase in yolk lightness. It was thus expected that the yolks of eggs laid by older hens would be lighter (paler) than younger hen eggs as storage time progress. However, this assumption was inconclusive, as the rate of change could not be determined for each hen age due to the large variation in values during the 90 days storage period. Although, from Table 4.8 it appears that eggs laid by older hens showed paler yolks at the end of the 90-day storage interval (69 weeks = 51.46; 77 weeks = 52.40) compared to younger hen ages (21 weeks = 44.22; 29 weeks = 45.18). This suggests that eggs laid by younger hens showed darker egg yolks compared to older hens when stored for 90 days. Yolk colour (L*) can be defined as the reflectance of light from an object (Nys, 2000). It has already been established that during storage, eggs laid by older hens displayed a greater increase in yolk size and bigger incidence of yolk rupturing due to a thinner vitelline membrane. It is thus likely that more light reflected from eggs laid by older hens due to a thinner vitelline membrane with increased storage compared to eggs laid by younger hens with a possible thicker vitelline membrane when the colour spectrophotometer measurements

were taken. This would explain why eggs laid by younger hens displayed darker yolks compared to eggs laid by older hens (paler yolks) as storage time increased (Table 4.8).

Yolk colour b* (yellowness) on the other hand showed an overall increase between 0 and 30 days of storage, but declined thereafter between all hen ages as storage time progressed (Table 4.9). Research done on yolk colour b* over the years are quite controversial, as some studies reported an increase in yolk yellowness with increased storage (Bhale *et al.*, 2003), while others reported a decrease in yolk yellowness (Barreras Serrano *et al.*, 2016). None of these studies could explain why these results, in terms of yolk yellowness, were obtained. The increase in yellowness in the current study can possibly be ascribed to the increase in yolk lightness, also observed within the first few days of storage (Table 4.8). This was consistent with Bhale *et al.* (2003), who reported that a five week storage experiment resulted in increase in yolk yellowness. The decrease in yellowness after 30 days of storage might also be a result of a decrease in the carotenoid content due to oxidation (Britton, 1995), however this assumption cannot be said for certain as the carotenoid content was not measured. It is shown in Table 4.9 that the interaction between hen age and storage time had a significant effect ($P<0.01$) on yolk colour b*. As previously stated, yolk colour L* was lower (darker) in yolks of eggs laid by young hens compared to older hens. This would explain why older hens showed yolks higher in the yellowness as storage time increased, as their yolks were not as dark as younger hen's egg yolks. It is possible that there was higher light reflection from yolks in eggs laid by older hens (due to a thinner vitelline membrane), which might have contributed to more yellow yolks compared to eggs laid by younger hens as storage time progressed. Younger hens laid eggs with thicker vitelline membranes, which might have absorbed more light, contributing to darker and less yellow egg yolks. The rate of change could also not be determined for yolk yellowness due to varying values between hen ages over the 90 days storage period. It was thus not possible to establish if there was a greater decrease in yellowness in egg yolks laid by younger hens compared to that of older ones.

The interaction results between storage time and hen age in terms of the Roche colour fan is reported in Figure 4.3. To ease interpretation of the Roche yolk colour fan results, only Figure 4.3 was included and not the means with standard deviations table, which was used to summarize the previous parameters in Section 4.3.2. The erratic pattern made it impossible to calculate the rate of change for the different hen ages as time progressed. This made it difficult to identify which hen age had the greatest rate of change for the Roche yolk colour fan. It was noted that colour measurement (L*, a* and b*) gave more reliable readings between hen ages with increased storage time compared to that of the Roche colour fan score. The oscillating measurements of the Roche colour fan (Figure 4.3) can be attributed to the human eye, which cannot interpret three different colour measurements at the same time as compared to the spectrophotometer (Vuilleumier, 1969). The human eye can only interpret

Table 4.8 The means and standard deviations of yolk colour L* (lightness) for eggs stored at different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)					
	0	15	30	45	60	90
21	49.79^{p} ± 1.66	54.58^{bcdefg} ± 1.40	53.92^{cdefghi} ± 1.36	49.88^{p} ± 1.47	47.77^{r} ± 1.71	44.22^{t} ± 1.03
29	51.57^{mn} ± 1.32	55.33^{ab} ± 1.19	55.13^{abcd} ± 1.71	52.91^{ijkl} ± 1.59	49.51^{p} ± 1.77	45.18^{t} ± 0.99
37	51.54^{mn} ± 1.73	55.03^{abcd} ± 1.45	55.09^{abcde} ± 1.72	52.85^{ijkl} ± 1.78	49.25^{pq} ± 1.56	45.26st ± 1.21
45	51.69^{lmn} ± 1.63	55.49^{ab} ± 1.14	54.86^{abcde} ± 1.62	52.68^{ijklm} ± 1.52	49.27^{pq} ± 1.34	46.72^{rs} ± 1.23
53	51.72^{lmn} ± 1.83	54.78^{abcdef} ± 1.50	55.03^{abcde} ± 0.95	53.75^{defghij} ± 1.08	50.05^{op} ± 1.36	47.60^{qr} ± 1.08
61	51.66^{lmn} ± 1.21	55.87^{a} ± 1.07	55.08^{abcde} ± 1.40	53.59^{fghij} ± 1.14	51.68^{lmn} ± 1.35	50.69^{nop} ± 0.87
69	51.83^{lmn} ± 1.43	55.90^{a} ± 1.53	54.49^{bcdefgh} ± 1.46	53.20^{hijk} ± 1.40	52.71^{ijklm} ± 1.53	51.46^{lmno} ± 1.28
77	52.05^{klmn} ± 1.16	55.99^{a} ± 1.37	55.20^{abc} ± 1.04	53.78^{efghij} ± 0.98	53.31^{ghijk} ± 1.33	52.40^{jklm} ± 1.44
Source of variance						
Storage time	*<0.01*					
Hen age	*<0.01*					
Storage time × Hen age	*<0.01*					

Table 4.9 The means and standard deviations of yolk colour b* (yellowness) for eggs stored at different time intervals and laid by different aged hens

Hen Ages (Weeks)	Storage Time (Days)					
	0	15	30	45	60	90
21	46.85^{pq} ± 1.88	49.39^{no} ± 1.84	54.46^{bcde} ± 1.59	53.13^{efghi} ± 1.85	52.21^{ghijkl} ± 1.48	51.50^{jkl} ± 1.79
29	45.68^{qr} ± 1.83	49.01^{no} ± 1.67	54.20^{bcde} ± 1.57	53.89^{cdef} ± 1.31	52.09^{hijkl} ± 1.70	51.64^{jkl} ± 1.83
37	45.86^{qr} ± 1.78	45.61^{qr} ± 1.97	55.49^{ab} ± 2.34	55.14^{abcd} ± 1.83	52.37^{fghijkl} ± 1.70	51.16^{klm} ± 1.58
45	46.70^{pq} ± 2.01	44.00^{s} ± 1.57	56.27^{a} ± 2.03	55.22^{abcd} ± 2.39	53.68^{defgh} ± 1.72	51.04^{lm} ± 2.13
53	46.94^{pq} ± 1.52	44.34^{rs} ± 1.23	53.68^{defgh} ± 0.84	53.56^{defgh} ± 0.83	53.33^{efghi} ± 1.87	53.16^{defghijk} ± 0.93
61	46.81^{pq} ± 1.70	46.82^{pq} ± 1.38	53.93^{cdef} ± 1.66	53.75^{defg} ± 2.21	53.91^{bcdef} ± 1.02	52.32^{fghijkl} ± 1.37
69	46.74^{qp} ± 1.6	48.14^{op} ± 1.72	54.40^{bcde} ± 1.27	54.04^{bcdef} ± 1.54	53.55^{defghi} ± 1.90	51.91^{ghijkl} ± 1.18
77	46.07^{q} ± 1.89	49.74^{mn} ± 1.83	55.45^{abc} ± 1.89	55.13^{abcd} ± 1.43	53.17^{efghij} ± 2.11	52.99^{efghijk} ± 2.20
Source of variance						
Storage time	*<0.01*					
Hen age	*0.02*					
Storage time × Hen age	*<0.01*					

colour in three dimensions, which include lightness (distinguishing between light and dark) and two chromatic attributes known as hue (colour perception such as yellow, green, etc.) and chroma (purity of the colour) (Ozakar Ilday *et al.*, 2014). The Roche colour fan is a combination of colours (L*, a* and b*) which make it difficult for humans to distinguish between different yolk colours. The L*, a* and b* colour measurements are accurate because they measure each colour on the colour axes according to the Hunter colour scale as seen in Figure 3.2 (Hunter, 1958). This would explain the fluctuating measurements taken by the yolk colour fan seen in Figure 4.3. It is important to note that measurements taken by the Roche colour fan can be influenced by different light intensities. This can lead to human errors occurring during measurement (Narinç *et al.*, 2015). Another dilemma with the use of the Roche colour fan is the limited number of colour classes (1 - 16), which makes it possible for the user to classify the yolk colour only when it is close to one of the classes. This method is faster and more user friendly, but the precision of the recordings can easily be impaired by external factors, such as light and user errors. Similar results were obtained by Hinton (1973), stating that it was difficult to distinguish between eggs in terms of their yolk colour using the more subjective Roche colour fan method compared to colour measurements based on the L*, a* and b* scale.

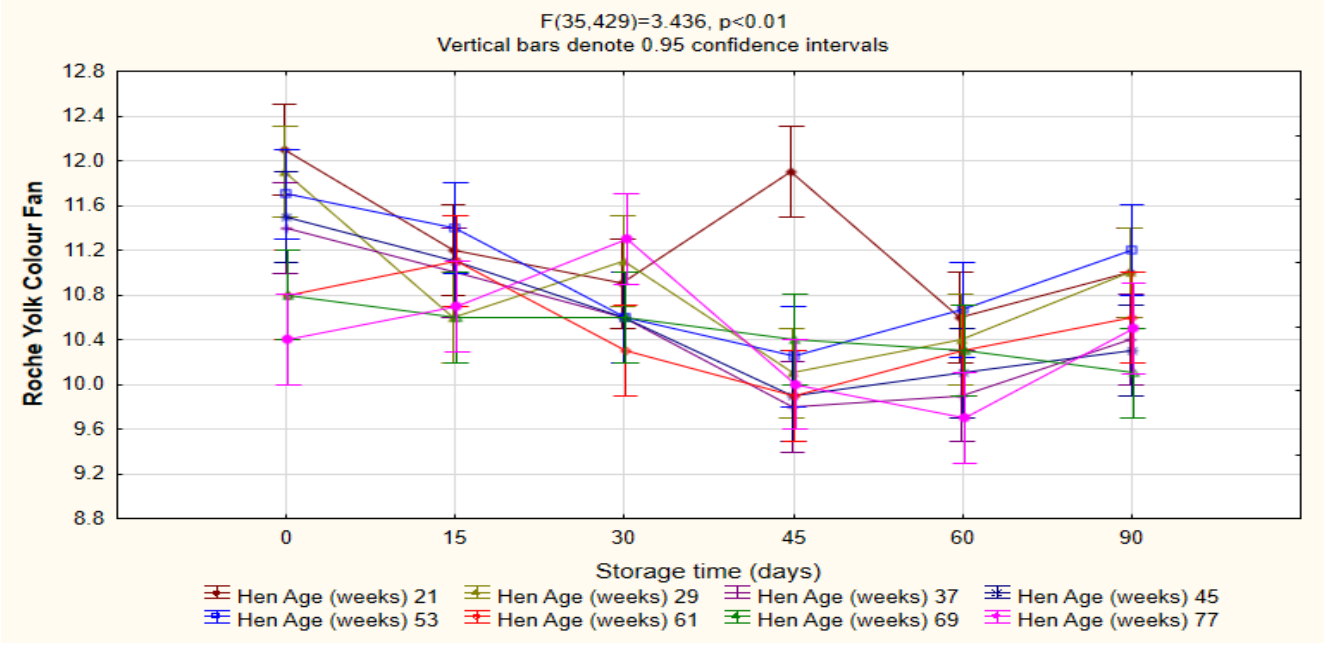


Figure 4.3 Graph illustrating the change in the Roche yolk colour fan score of eggs stored for 90 days, laid by Amberlink hens with ages ranging from 21 to 77 weeks.

4.3.3 Influence of storage time and hen age on proximate composition

For this section focus was placed on storage time and hen age's (main effects) interaction on the proximate composition of eggs. No significant interactions (P>0.05) were observed between all the proximate parameters (Table 4.10). The lipid and ash content were also unaffected by the main effect, storage time (P = 0.77 and P = 0.18, respectively). However, the moisture and protein content

were significantly affected by the main effect storage time, both obtaining a P-value less than 0.01 (Table 4.10).

The significant decrease in the moisture content, observed in Table 4.10, can be attributed to the evaporation of moisture from the albumen, through the eggshell pores to the surrounding environment, as explained in Section 4.3.1. These results coincide with numerous studies done over the years (Benton & Brake, 1996; Tilki & Saatci, 2004; Dudusola, 2009; Chung & Lee, 2014).

Table 4.10 The means ± standard deviations of proximate composition for eggs stored at different time periods ranging from 0 to 90 days

			Dry Matter Basis		
	Parameter	Moisture (%)	Protein (%)	Lipids (%)	Ash (%)
Treatment (Storage Time in Days)	**0**	$74.37^{a} \pm 1.98$	$49.84^{a} \pm 1.98$	37.47 ± 1.83	3.54 ± 0.27
	15	$72.63^{b} \pm 2.09$	$49.76^{a} \pm 1.92$	37.48 ± 1.83	3.59 ± 0.30
	30	$72.27^{b} \pm 1.80$	$49.49^{ab} \pm 1.97$	37.24 ± 1.56	3.49 ± 0.25
	45	$71.55^{c} \pm 1.67$	$49.38^{ab} \pm 1.93$	37.24 ± 1.56	3.57 ± 0.33
	60	$71.06^{d} \pm 1.31$	$49.13^{bc} \pm 1.98$	37.20 ± 1.65	3.53 ± 0.22
	90	$70.43^{e} \pm 1.86$	$48.72^{c} \pm 1.99$	37.30 ± 1.73	3.57 ± 0.20
	P value	<0.01	<0.01	0.77	0.18

($^{a, b, c, d, e}$) Means with different superscripts within the same column differ significantly (P≤0.05).

Table 4.10 shows that storage time had a significant effect on the protein content of eggs (P<0.01). It is important to note that the protein content was determined on a dry matter basis. It is concluded in the current study that moisture content decreased with an increase in storage time. A decrease in the moisture content will lead to an increase in the protein content, but only on a wet basis (as is basis) as the solid content of an egg increase with a decrease in moisture (Ahn *et al.*, 1997). It is shown in Table 4.10 that there was a slight decrease in the protein content (dry matter basis), as storage time progressed (from 49.84% on day 0 to 48.72% on day 90). The decrease in protein content could possibly be attributed to change in the lysozyme-ovomucin complex. In Section 4.3.1 it was mentioned that stored eggs could experience an increase in albumen pH due to an increase conversion of carbon dioxide to carbonate. The increase in pH, as storage time progress, later exceeds the isoelectric point (9.2) of the lysozyme-ovomucin complex, resulting in the complete dissociation of this protein (Hayakawa *et al.*, 1983; Yamamoto & Gutierrez, 1997). Omana *et al.* (2011) on the other hand concluded that protein degradation during storage might be caused by a combination of increase in pH and proteolysis caused by hydrolytic enzymes (protease). There are various hydrolytic enzymes present in the albumen and it is possible that an increase in pH, during storage, could have increased the hydrolytic enzyme activity, but unfortunately it cannot be said for certain, as neither the pH nor the hydrolytic enzyme activity was measured in the current study. However, previous studies reported that the protease activity is optimum at a pH between 9 and 11 (Ellaiah *et al.*, 2002) and due to the shift in albumen pH from relatively neutral to more basic, with

increased storage time (Silversides & Budgell, 2004; Jin *et al.*, 2011), it is likely that the protease activity increase resulting in a decrease in the protein content (Table 4.10). It is thus recommended that future egg storage studies place emphasis on how the lysozyme-ovomucin complex changes during storage and to what extent the complex, as well as the hydrolytic enzymes, are influenced by changes in the albumen pH.

4.4 Conclusion

The aim of the current study was to explore the effect of hen age and storage time on certain egg quality parameters. The main effect (storage time) had a significant effect on egg weight, shell weight, shell thickness, albumen weight, yolk weight, yolk colour a* and vitelline membrane integrity. Whole egg weight decreased with increased storage time due to the direct correlation to albumen weight. Albumen weight decreased due to loss in moisture to the storage environment through evaporation and to the yolk through osmosis, which would explain the increase in the yolk weight. Increased storage time led to a decrease in shell weight and shell thickness, which is likely due to drying of the shell membranes resulting in shrinkage and deterioration. Storage time significantly increased yolk redness, which could possibly be ascribed to change in the carotenoid structure due to oxidation. The incidence of rupturing egg yolks also increased, likely due to stretching of the vitelline membrane with increase in yolk size.

There were however several significant interactions between hen age and storage time with regards to certain egg quality parameters. Eggs laid by older hens displayed bigger yolks with thinner vitelline membranes, resulting in easier accumulation of moisture. This led to a less rigid structure and thus a greater decrease in yolk height compared to the yolks of eggs laid by younger hens. The rate of change suggested there was a greater decrease in thick albumen height in eggs laid by older hens than younger ones. This change was not observed in the thin albumen height, suggesting greater decrease in thick albumen height compared to thin albumen height as storage time increased. The thick albumen height is directly related to HU. It was therefore not surprising that the HU demonstrated greater decrease in eggs laid by older hens compared to younger ones. The HU results indicated that eggs laid by younger hens can be stored for longer periods of time when compared to the HU of eggs laid by older hens. The HU was negatively correlated to the thick albumen spreading distance. There was a greater increase of thick albumen spreading in eggs laid by older hens compared to that of younger hens. However, the same was not true for the thin albumen spreading. The results indicated that eggs laid by older hens had a brighter more yellowish colour after the storage duration, whilst eggs laid by younger hens were less yellow in colour and darker after 90 days of storage. The changes observed in yolk colour could be due to increased oxidation resulting in the alteration of the carotenoid structure. It could also be due to more light reflection from eggs laid by older hens due to a thinner vitelline membrane. It is advised that future research should focus on investigating the relationship between storage time and the carotenoid content as well as the effect of a thinning vitelline membrane on yolk colour measurements, as

research on this topic is limited. The inability of the human eye to interpret three different colour measurements led to inconsistent results, in terms of the Roche yolk colour fan over the 90-day storage period. This raises the question if the Roche yolk colour fan is a viable technique to determine colour.

There were no significant interactions between any of the proximate parameters. However, storage time significantly decreased the moisture and protein content. Additional research is necessary to investigate the relationship between the pH and the lysozyme-ovomucin complex, as well as the relationship between the pH and the hydrolytic enzymes present in the albumen with increased storage duration This could assist with evaluating if these factors might contribute to the change noted in the protein content.

The interaction of the main effects, storage time and hen age had a significant effect on several of the egg quality parameters. Storage time on its own, also had substantial effects on most of the egg quality parameters. Since most egg quality parameters already showed noteworthy change within the first 15 days of storage, it might be of value for future studies to shorten the storage period and increase the frequency of analysis. Storage time can have adverse effects on most egg quality parameters and even more detrimental outcomes in combination with hen age.

4.5 References

Adamiec, J., Dolezal, M., Mikova, K., & Davidek, J. 2018. Changes in egg volatiles during storage. Czech J. Food Sci. 20, 79–82.

Ahn, D. U., Kim, S. M., & Shu, H. 1997. Effect of egg size and strain and age of hens on the solids content of chicken eggs. Poult. Sci. 76, 914–919.

Akter, Y., Kasim, A., Omar, H., & Sazili, A. Q. 2014. Effect of storage time and temperature on the quality characteristics of chicken eggs. J. Food, Agric. Environ. 12, 87–92.

Akyurek, H., & Okur, A. A. 2009. Effect of storage time, temperature and hen age on egg quality in free-range layer hens. J. Anim. Vet. Adv. 8, 1953–1958.

Altan, Ö., Akbaş, Y., Sevgican, F., & Erkek, R. 1997. Effects of cold storage on egg quality. . Turkish J. Vet. Anim. Sci. 21, 335–339.

Barbosa, V. C., Gaspar, A., Calixto, L. F. L., & Agostinho, T. S. P. 2011. Stability of the pigmentation of egg yolks enriched with omega-3 and carophyll stored at room temperature and under refrigeration. Rev. Bras. Zootec. 40, 1544–2011.

Barreras Serrano, A., Figueroa Saavedra, F., Sánchez López, E., Pérez Linares, C., & Juárez Cervantes, L. 2016. Temperature and shelving period effect on albumin height and color of supermarket eggs sold in northwest Mexico. Acta Univ. 26, 12–18.

Beardsworth, P. M., & Hernandez, J.-M. 2004. Yolk colour - An important egg quality attribute. Int. Poult. Prod. 12, 17–18.

Benton, C. E., & Brake, J. 1996. The effect of broiler breeder flock age and length of egg storage on egg albumen during early incubation. Poult. Sci. 75, 1069–1075.

Bhale, S., Prinyawiwatkul, H. K., Farr, A. J., Nadarajah, K., & Meyers, S. P. 2003. Chitosan coating improves shelf life of eggs. J. Food Sci. 68, 2378–2383.

Brake, J., Walsh, T., Benton, C., & Petitte, J. 1997. Egg handling and storage. Poult. Sci. 76, 144–151.

Britton, G. 1995. Structure and properties of carotenoids in relation to function. FASEB J. 9, 1551–1558.

Britton, G., & Khachik, F. 2009. Carotenoids in Food. Carotenoids, 45–65.

Burley, R. W., & Vadehra, D. V. 1989. The avian egg : chemistry and biology. Wiley.

Callejo Ramos, A., Cardoso Maciel, W., Daza Andrada, A., Siqueira de Castro Teixeira, R., & Buxade Carbo, C. 2010. Effect of bird age and storage system on physical properties of eggs from brown laying hens. PUBVET, Publicaçoes em Med. Vet. e Zootec. 4, 961–971.

Caner, C. 2005. The effect of edible eggshell coatings on egg quality and consumer perception. J. Sci. Food Agric. 85, 1897–1902.

Christensen, V. L., Grimes, J. L., Wineland, M. J., & Bagley, L. G. 2001. Effects of turkey breeder hen age, strain, and length of the incubation period on survival of embryos and hatchlings. Poult. Sci. Assoc. Inc. 10, 5–15.

Chung, S. H., & Lee, K. W. 2014. Effect of hen age, Storage duration and temperature on egg quality in laying hens. Int. J. Poult. Sci. 13, 634–636.

Curtis, P. A., Kerth, L. K., & Anderson, K. E. 2005. Quality and compositional characteristics of layer hens as affected by bird age.

Dawes, C. M. 1975. Acid-base relationships within the avian egg. Biol. Rev. Camb. Philos. Soc. 50, 351–371.

Decuypere, E., Tona, K., Bruggeman, V., & Bamelis, F. 2001. The day-old chick: a crucial hinge between breeders and broilers. Worlds. Poult. Sci. J.

Demirel, Ş., & Kirikçi, K. 2009. Effect of different egg storage times on some egg quality characteristics and hatchability of pheasants (Phasianus colchicus). Poult. Sci. 88, 440–444.

Dudusola, I. 2009. Effects of storage methods and lenght of storage on some quality parameters of japanese quail eggs. Tropicultura 27, 45–48.

Eke, M. O., Olaitan, N. I., & Ochefu, J. H. 2013. Effect of storage conditions on the quality attributes of shell (table) eggs. Niger. Food J. 31, 18–24.

Ellaiah, P., Srinivasulu, B., & Adinarayana, K. 2002. A review on microbial alkaline proteases. J. Sci. Ind. Res. (India). 61, 690–704.

Gavril, R., & Usturoi, M. G. 2012. Effects of temperature and storage time on hen eggs quality. J. Biotechnol. 161, 40.

Gennadios, A., Handa, A., Froning, G. W., Weller, C. L., & Hanna, M. A. 1998. Physical properties of egg white-dialdehyde starch films J. Agric. Food Chem 46, 1297–1302.

Grashorn, M. 2016. Effects of storage conditions on egg quality. 50, 23–25.

Haugh, R. . 1937. The Haugh unit of measuring egg quality. US Poult. Mag 43, 522–555, 572–573.

Hayakawa, S., Kondo, H., Nakamura, R., & Sato, Y. 1983. Effect of β-ovomucin on the solubility of α-ovomucin and further inspection of the structure of ovomucin complex in thick egg white. Agric. Biol. Chem. 47, 815–820.

Hill, A. T., & Hall, J. W. 1980. Effects of various combinations of oil spraying, washing, sanitizing, storage time, strain, and age of layer upon albumen quality changes in storage and minimum sample sizes required for their measurement. Poult. Sci. 59, 2237–2242.

Hinton, C. F. 1973. Reflectance colorimetric evaluation of carotenoid pigment deposition in broilers and egg yolks. 34, 1–89.

Hunter, R. 1958. Photoelectric color difference meter*. J. Opt. Soc. Am. 48, 985.

Jacob, J. P., Miles, R. D., & Mather, F. Ben. 2000. Egg quality 1. Anim. Sci., 1–12.

Jacob, J. P., Wilson, H. R., Miles, R. D., Butcher, G. D., & Mather, F. B. 2003. Factors affecting egg production in backyard chicken flocks. EDIS Publ. Syst., 1–8.

Jin, Y. H., Lee, K. T., Lee, W. I., & Han, Y. K. 2011. Effects of storage temperature and time on the quality of eggs from laying hens at peak production. Asian-Australasian J. Anim. Sci. 24, 279–284.

Jones, D. R. 2007. Egg functionality and quality during long-term storage. Int. J. Poult. Sci. 6, 157–162.

Jones, D. R., & Musgrove, M. T. 2005. Effects of extended storage on egg quality factors. Poult. Sci. 84, 1774–1777.

Kaspers, B. 2016. An egg a day – the physiology of egg formation. Lohmann Inf. 50, 13–15.

Kato, A., Nakamura, R., & Sato, Y. 1970. Studies on changes in stored shell eggs. nihon chikusan gakkaiho 34, 1009–1013.

Kemps, B. J., Bamelis, F. R., Mertens, K., Decuypere, E. M., de Baerdemaeker, J. G., & de Ketelaere, B. 2010. The assessment of viscosity measurements on the albumen of consumption eggs as an indicator for freshness. Poult. Sci. 89, 2699–2703.

Khan, M. J. A., Khan, S. H., Bukhsh, A., Amin, M., Khan, M. J. A., Khan, S. H., Bukhsh, A., Amin Khan, M., & Amin, M. 2014. The effect of storage time on egg quality and hatchability characteristics of Rhode Island Red (RIR) hens Vet. Arh. 84, 291–303.

Kirunda, D. F. K., & McKee, S. R. 2000. Relating quality characteristics of aged eggs and fresh eggs to vitelline membrane strength as determined by a texture analyzer. Poult. Sci. 79, 1189–1193.

Kul, S., & Seker, I. 2004. Phenotypic correlations between some external and internal egg quality traits in the Japanese quail (Coturnix coturnix japonica). Int. J. Poult. Sci.

Lapão, C., Gama, L. T., & Soares, M. C. 1999. Effects of broiler breeder age and length of egg storage on albumen characteristics and hatchability. Poult. Sci.

Leeson, S., & Caston, L. J. 1997. A Problem with characteristics of the thin albumen in laying hens. Poult. Sci. 76, 1332–1336.

Lewis, N. M., Seburg, S., & Flanagan, N. L. 2000. Enriched eggs as a source of N-3 polyunsaturated fatty acids for humans. Poult. Sci. 79, 971–974.

Lewko, L., & Gornowicz, E. 2009. Egg albumen quality as affected by bird origin. J. Cent. Eur. Agric. 10, 455–464.

McClements, D. J., & Decker, E. A. 2000. Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. J. Food Sci. 65, 1270–1282.

McNally, E. H. 1943. The origin and structure of the vitelline membrane of the domestic fowl's egg. Poult. Sci. 22, 40–43.

Messens, W., Grijspeerdt, K., & Herman, L. 2005. Eggshell characteristics and penetration by Salmonella enterica serovar Enteritidis through the production period of a layer flock. Br. Poult. Sci. 46, 694–700.

Monira, K. N., Salahuddin, M., & Miah, G. 2003. Effect of breed and holding period on egg quality characteristics of chicken. Int. J. Poult. Sci. 2, 261–263.

Narinç, D., Aygün, A., Küçükönder, H., Aksoy, T., & Gürcan, E. K. 2015. An application of bootstrap technique in animal science: Egg yolk color sample. KafKas Univ. Vet. Med. J. 21, 631–637.

Nascimento, B. B. J., Ozorio, L. P., Rezende, C. M., Soares, A. G., & Fonseca, M. J. de O. 2008. Ciência e tecnologia de alimentos. Food Sci. Technol, Campinas 28, 649–658.

Nys, Y. 2000. Dietary carotenoids and egg yolk coloration - A review. Arch. fur Geflugelkd. 64, 45–54.

Omana, D. A., Liang, Y., Kav, N. N. V., & Wu, J. 2011. Proteomic analysis of egg white proteins during storage. Proteomics 11, 144–153.

Ozakar Ilday, N., Celik, N., Bayindir, Y. Z., & Seven, N. 2014. Effect of water storage on the translucency of silorane-based and dimethacrylate-based composite resins with fibres. J. Dent. 42, 746–752.

Pamarin, W., Garcia, K. M., No, H. K., Prinyawiwatkul, W., & Ingram, D. R. 2009. Selected quality and shelf life of eggs coated with mineral oil with different viscosities. J. Food Sci. 74, 423–429.

Raji, a O., Aliyu, J., Igwebuike, J. U., & Chiroma, S. 2009. Effect of storage methods and time on egg quality traits of laying hens in a hot dry climate. ARPN J. Agric. Biol. Sci. 4, 1–7.

Reis, L. H., Gama, L. T., & Chaveiro Soares, M. 1997. Effects of short storage conditions and broiler breeder age on hatchability, hatching time, and chick weights. Poult. Sci. 76, 1459–1466.

Robert, J. R. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. J. Poult. Sci. 41, 161–177.

Roberts, : J R, & Ball, W. 2005. Egg quality guidelines for the Australian egg industry. Eur. Symp. Qual. Eggs Egg Prod. 3.

Rodríguez-Navarro, A. B., Domínguez-Gasca, N., Muñoz, A., & Ortega-Huertas, M. 2013. Change in the chicken eggshell cuticle with hen age and egg freshness. Poult. Sci. 92, 3026–3035.

Samli, H. E., Agma, A., & Senkoylu, N. 2005. Effects of storage time and temperature on egg quality

in old laying hens. J. Appl. Poult. Res. 14, 548–553.

Seymour, R. S., & Piiper, J. 1988. Aeration of the shell membranes of avian eggs. Respir. Physiol. 71, 101–115.

Silversides, F. G., & Budgell, K. 2004. The relationships among measures of egg albumen height, pH, and whipping volume. Poult. Sci.

Silversides, F. G., & Scott, T. A. 2001. Effect of storage and layer age on quality of eggs from two lines of hens. Poult. Sci. 80, 1240–1245.

Song, K. T., Choi, S. H., & Oh, H. R. 2000. A comparison of egg quality of pheasant, chukar, quail and guinea fowl. asian-australasian J. Anim. Sci. 13, 986–990.

Stadelman, W. J. 1986. The preservation of quality in shell eggs. egg science and technology. CRC Press, 63–73.

Surai, P. F., Speake, B. K., & Sparks, N. H. C. 2001. Carotenoids in avian nutrition and embryonic development. 2.Antioxidant properties and discrimination in embryonic tissues. J. Poult. Sci. 38, 1–27.

Tilki, M., & Saatci, M. 2004. Effects of storage time on external and internal characteristics in partridge (Alectoris graeca) eggs. Rev. Med. Vet. (Toulouse). 155, 561–564.

Tona, K., Onagbesan, O., De Ketelaere, B., Decuypere, E., & Bruggeman, V. 2004. Effects of age of broiler breeders and egg storage on egg quality, hatchability, chick quality, chick weight, and chick posthatch growth to forty-two days. J. Appl. Poult. Res. 13, 10–18.

Toussant, M. J., & Latshaw, J. D. 1999. Ovomucin content and composition in chicken eggs with different interior quality. J. Sci. Food Agric. 79, 1666–1670.

Tsai, W. T., Yang, J. M., Lai, C. W., Cheng, Y. H., Lin, C. C., & Yeh, C. W. 2006. Characterization and adsorption properties of eggshells and eggshell membrane. Bioresour. Technol. 97, 488–493.

Vuilleumier, J. P. 1969. The ‘Roche yolk colour fan‘—An instrument for measuring yolk colour. Poult. Sci. 48, 767–779.

Walsh, T. J., Rizk, R. E., & Brake, J. 1995. Effects of temperature and carbon dioxide on albumen characteristics, weight loss, and early embryonic mortality of long stored hatching eggs. Poult. Sci.

Wellman-Labadie, O., Picman, J., & Hincke, M. T. 2008. Antimicrobial activity of the Anseriform outer eggshell and cuticle. Comp. Biochem. Physiol. - B Biochem. Mol. Biol. 149, 640–649.

Williams, K. C. 1992. Some factors affecting albumen quality with particular reference to Haugh unit score. Worlds. Poult. Sci. J. 48, 5–16.

Williams, C. A., & Pieterse, E. 2019. The effect of Nutrifen® and Nutrifen Plus® in the diet of Hy-Line layers on production, egg quality and egg shelf life. , 50–73.

Yamamoto, T., & Gutierrez, M. A. 1997. Nutritive Evaluation of Hen Eggs, 25–35.

Yetim, H., & Ockerman, H. W. 1995. The effects of egg white, tumbling and storage time on proximate composition and protein fractions of restructured fish product. J. Aquat. Food Prod. Technol. 4, 65–78.

Chapter 5
Investigating the application of near infrared (NIR) hyperspectral imaging for differentiation and quantification of hen egg quality

Abstract

Near infrared (NIR) hyperspectral imaging was used to distinguish between eggs laid by different aged hens and between eggs stored for different time intervals. Eggs were collected from eight different hen age groups ranging from 21 to 77 weeks with each consecutive age group eight weeks older than the previous. Eggs were stored for six storage intervals including 0 (day egg was laid and collected), 15, 30, 45, 60 and 90 days. Quality analysis as well as hyperspectral imaging took place on each successive storage interval. Near infrared hyperspectral images for all eggs were acquired with a HySpex SWIR-384 pushbroom imaging system in the 750 – 2500 nm spectral range. Principal component analysis (PCA) was carried out to explore if eggs could be separated based on hen age and based on storage period. To improve interpretation of results, eggs from day 0 from all hen age groups were analysed together to explore trends. Secondly, eggs from 21-week-old hens were analysed over the full storage period to explore patterns. The application of standard normal variate (SNV) preprocessing showed the best separation when used for both analyses. Proximate data, determined on a wet chemical basis, was used to evaluate if differences observed, explain the trends observed in the PCA plots. A separation between young hen ages (21, 29 and 37 weeks) and older hen ages (69 and 77 weeks) was detected and the differences between the age groups could be attributed to high moisture and low lipid content in eggs laid by younger hens. The opposite was recorded in eggs laid by older hens. Principal component analysis proved useful in separating eggs stored for different time periods, based on their proximate composition. Separation or clustering occurred between eggs stored for 0 days, 15 days and longer storage periods (45, 60 and 90 days). Eggs stored for shorter periods displayed a higher moisture and lower lipid content. The opposite was recorded for eggs stored for longer durations. Subsequently, partial least squares regression (PLSR) was performed to construct quantitative prediction models for the Haugh unit as well as the protein, lipid and moisture content. The raw NIR data was preprocessed with various techniques to optimize the model conditions during cross validation. The best parameters (preprocessing and number of principal components) were used to evaluate the prediction accuracy. Several steps were implemented to improve the models including the comparison of images of the whole egg *versus* a smaller region of interest or ROI (40 x 40 pixel size). This was followed by testing wavelength reduction by removing the end regions of the spectra (952 - 1011 nm and 2403 - 2517 nm) to investigate if the models improved. Models constructed with the whole egg spectra with a reduction in the wavelength range gave superior results for all models. The HU model gave the most favorable

results (R^2 = 0.76; RMSEP = 14.13; RPD = 2.00), while protein, lipid and moisture models gave less accurate models (R^2 = 0.64; RMSEP = 2.35; RPD = 1.66), (R^2 = 0.64; RMSEP = 2.97; RPD = 1.66) and (R^2 = 0.68; RMSEP = 6.04; RPD = 1.76) respectively.

Keywords: *hypercube, hyperspectral imaging, partial least squares regression, preprocessing, principal component analysis, spectroscopy, wavebands*

5.1 Introduction

Eggs are an excellent source of proteins, essential vitamins and minerals. In South Africa, eggs are considered to be the most affordable and obtainable animal protein source, making eggs one of the most important foods in the country (SAPA, 2017). Proteins consists out of different amino acid combinations, 20 of which are important for humans to synthesize an extensive range of proteins. The human body can produce 11 of these 20 amino acids via transaminase, but the remaining essential amino acids have to be obtained from our diet, and these are all present in eggs (Greenblum, 2008). The nutritional value of eggs is susceptible to internal and external changes, which are mainly influenced by the hen's age and storage conditions (Stadelman & Cotterill, 1977). Unfavourable changes can lead to a reduction in egg quality and an overall decline in consumers' acceptance of the product (Jones & Musgrove, 2005).

In February 2019, South Africa produced an average of 422 700 eggs per week and it is estimated to increase by 10.6% at the end of 2020 (SAPA, 2019). Due to an increase in price of other proteins such as meat, a movement toward the consumption of eggs occurred, which is leading to an increased egg demand. In 2017, the average person consumed 6.61 kilograms (kg) eggs in South Africa, 15.57 kg in America and up to 22.72 kg in China (Weindl *et al*., 2015). Due to this high demand, South Africa is also a net exporter of eggs, with roughly 3800 ton eggs per year being exported, which contributes greatly to the country's economy (Department of Agriculture, Forestry and Fisheries, 2017). It is thus important to maintain a high egg quality and reduce egg spoilage to sustain the consumers' demands and nutritional requirements.

The freshness of eggs is determined by examining the albumen quality. For the past eight decades the Haugh Unit (HU) has been the preferred method used by industry and regulators to determine the freshness of eggs (Haugh, 1937). The HU is calculated by using a logarithm of the height of the thick albumen adjusted to the total egg weight. The HU has a range from 0 to 130, where higher values are associated with fresher eggs and as the value decreases, so does the freshness (Eisen *et al*., 1962; Zhao *et al*., 2010). Emphasis should be placed on investigating various factors that can influence the quality of eggs. The most important factors that are used to evaluate egg quality is not only the albumen height, but also the chemical composition of the egg, which is influenced by hen age and storage duration (Stadelman & Cotterill, 1977).

Over the years, numerous studies have been done to analyse the effect of hen age on certain internal egg quality characteristics. Results of these studies and findings of the current study have indicated that hen age significantly affects these characteristics (Chapter 3). These internal

characteristics can include protein, moisture and lipid content. Increasing hen age leads to an increase in the yolk and albumen weight (Silversides & Scott, 2001), which is directly correlated to the amount of protein, moisture and lipid content (Moran & Reinhart, 1980; OnbaŞılar *et al*., 2011). Scott & Warren (1941) first reported that an increase in hen age resulted in a higher lipid to protein ratio, which was later confirmed by Whitehead *et al*. (1991). The reason for this is in an egg, the yolk has the highest lipid content and the albumen the highest protein content (OnbaŞılar *et al*., 2011).

As hens age, there is a greater proportional increase in the yolk weight than the albumen weight in eggs resulting in the increased lipid: protein ratio. Applegate *et al.* (1998) also found that there is a higher percentage moisture content in eggs laid by younger hens compared to older hens.

Storage time is another factor that can have a substantial negative impact on egg quality characteristics (Goodwin *et al*., 1962) as seen in Chapter 4. Increased storage time leads to a reduction of moisture and carbon dioxide. Albumen moisture is not only lost through the shell pores to the surrounding environment, but also from the albumen to the yolk, which results in higher yolk weight and lower albumen weight. Similar to hen age, storage time can also have a significant impact on the proximate composition of eggs (Meehan *et al*., 1962).

Application of conventional methods such as HU evaluation and quantification of protein, moisture and lipid content to evaluate egg quality parameters are expensive, tedious, time consuming and leads to the destruction of the sample (Abdel-Nour *et al*., 2011). These methods only permit the analysis of one or a few samples per batch. As a result, these methods only provide the average chemical composition and quality for a group of eggs, which has to represent the larger batch. A faster screening method is required for evaluating the internal composition and quality of individual eggs to ensure acceptable quality for human consumption. Near infrared (NIR) spectroscopy and hyperspectral imaging is a rapid method that could be used to accomplish these goals.

The combination of NIR spectroscopy and digital imaging to obtain spatial (x and y) and spectral (λ) information from a sample is known as NIR hyperspectral imaging (Workman, 1999; Osborne, 2000; Geladi *et al*., 2007; Fernández *et al*., 2012). The technique is used to gather information about the chemical and physical properties of an object. Combining NIR hyperspectral imaging with data analysis tools enable the development of methods to quantify certain chemical compounds and to investigate the chemical compound distribution throughout the sample (Burger & Geladi, 2006; Grahn & Geladi, 2007). NIR hyperspectral imaging captures images at several wavelengths in the NIR region (750 - 2500 nm) (Babin & Stramski, 2002; Yao *et al*., 2008). Each image contains the spectra for several data points (pixels) across the range of wavebands and this information is obtained within seconds of scanning. NIR hyperspectral imaging has diverse applications ranging not only from the food industry, but the agriculture sector as well (Lanza & Li, 1984; Givens *et al*., 1997; Cen & He, 2007; Ortiz-Somovilla *et al*., 2007; Abdel-Nour *et al*., 2011). Near infrared hyperspectral imaging and spectroscopy has been used to investigate the HU, bubble formation and scattered yolks in eggs (Zhang *et al*., 2015) as well as the protein, lipid and moisture

content of homogenised eggs (Zhao *et al.*, 2018). However, little research has been done to investigate the quality and internal composition of whole eggs affected by hen age as well as storage through means of exploratory analysis and quantitative predictions by implementing hyperspectral imaging.

The aim of the study was to assess the efficiency of hyperspectral imaging to differentiate between eggs laid by hens of different ages and eggs stored for different time periods respectively with the aid of PCA. After performing exploratory analysis, the application of NIR spectroscopy to construct PLSR models to predict the HU, protein, lipid and moisture content of eggs were investigated. Since hen age and storage period were shown to significantly impact the internal composition (Chapter 3 and Chapter 4), these two factors were taken into consideration and samples from varying hen age groups and storage periods were used to develop models. In addition, several approaches were implemented and the ability to improve the accuracy of the models were evaluated.

5.2 Materials and methods

5.2.1 Sample collection, preparation and storage

A total of 480 intact Amberlink hen eggs were used for the purpose of this trial. The eggs were collected from Rosendal Poultry Farm situated near Paarl (-33.738734, 19.029038). No eggs with cracked eggshells were collected. The eggs were placed in reinforced egg trays to prevent damage and was transported to the Department of Food Science (-33.925242, 18.871121), Stellenbosch University. Eggs were cleaned to remove any debris that could diminish the accuracy of the hyperspectral imaging process. Eggs were then subjected to hyperspectral imaging and afterwards transported to the Department of Animal Science (-33.931567, 18.867191) for quality investigation and proximate analysis. Eggs not analysed on the day of collection were stored to be analysed later (Chapter 4). To ensure the reliability and implementation of the models, a calibration and validation dataset consisting out of samples collected from eight different hen age groups (60 eggs per age group) and also stored for six storage intervals (80 eggs per storage group) were used/constructed to ensure a wide range of reference values. However, due to accidental breakage, some groups contained less samples, resulting in a total of 464 samples as displayed in Table 5.1. The youngest group of hens were 21 weeks and the oldest group 77 weeks with each consecutive age group, eight weeks older than the previous. Eggs from each age group were divided into six groups of ten eggs and were stored for different time intervals to test the effect of storage duration on certain egg chemical parameters. The storage intervals included 0, 15, 30, 45, 60 and 90 days where day 0 refers to the day the eggs were laid, which was the same day eggs were collected and analysed. Eggs were stored at the Department of Animal Science in a dark room where temperature was kept at 15.5˚C ± 2.2˚C and humidity at 75.8% ± 3.2%, which were subjected to hyperspectral imaging at each storage interval.

Table 5.1 Number of samples for each hen age and storage time group, used to set up the calibration and validation set

		Storage Time (in Days)						
		0	**15**	**30**	**45**	**60**	**90**	**Total**
Hen Age (in Weeks)	**21**	10	9	10	10	10	10	59
	29	10	10	10	9	10	8	57
	37	10	10	10	10	8	10	58
	45	10	8	10	9	10	10	57
	53	10	10	9	10	10	10	59
	61	10	10	10	10	10	10	60
	69	10	10	10	9	9	10	58
	77	9	9	9	9	10	10	56
	Total	79	76	78	76	77	78	**464**

5.2.2 NIR hyperspectral imaging system

Hyperspectral images were acquired using a HySpex short-wave infrared (SWIR-384) pushbroom imaging system (HySpex, Norsk Electro Optikk (NEO), Norway), equipped with a mercury-cadmium-telluride (MCT) detector (Figure 5.1). The system was controlled using the Breeze® (Prediktera AB, Umeå, Sweden) software version 2019.2.0, to regulate the motor speed, image acquisition and exposure time. All images were collected at 5.45 nanometer (nm) resolution intervals across a spectral range of 952 – 2517 nm resulting in 288 spectral bands. A 30 centimeter (cm) focal length lens producing a 95 mm field of view (FOV) was used. Two 150 W halogen lamps (Ushio lighting Inc., Japan) positioned 30 cm above the sample stage at a 45˚ angle, provided illumination in the 400 – 2500 nm spectral range. The frame rate was set to 100 hertz (Hz) with an exposure of 3 ms (milliseconds) to obtain good quality images. Before imaging commenced, the imaging system was turned on and left for 15 minutes for the SWIR system to cool down to -151 Kelvin (K). Lights were also given five minutes to warm up prior to imaging. A total of 464 intact eggs were scanned individually. When scanning, the eggs were placed on a round cap to keep it centered in the FOV on the conveyor belt, avoiding movement by the sample during imaging. A 50% grey Zenith Allucore diffuse reflectance standard (SphereOptics GmbH, Germany) served as a white reference. A dark reference was also recorded and both references were used to correct for uneven light intensity of different wavelength bands. Images produced a 323(x) x 384(y) x 288(λ) hypercube, when stacked together. The x and y dimensions provided the spatial data, used for creating the distribution map and the λ dimension generated spectral data, used for proximate and quality prediction. The images were radiometrically calibrated in the Breeze software prior to exporting the images in Envi format to the Evince processing software.

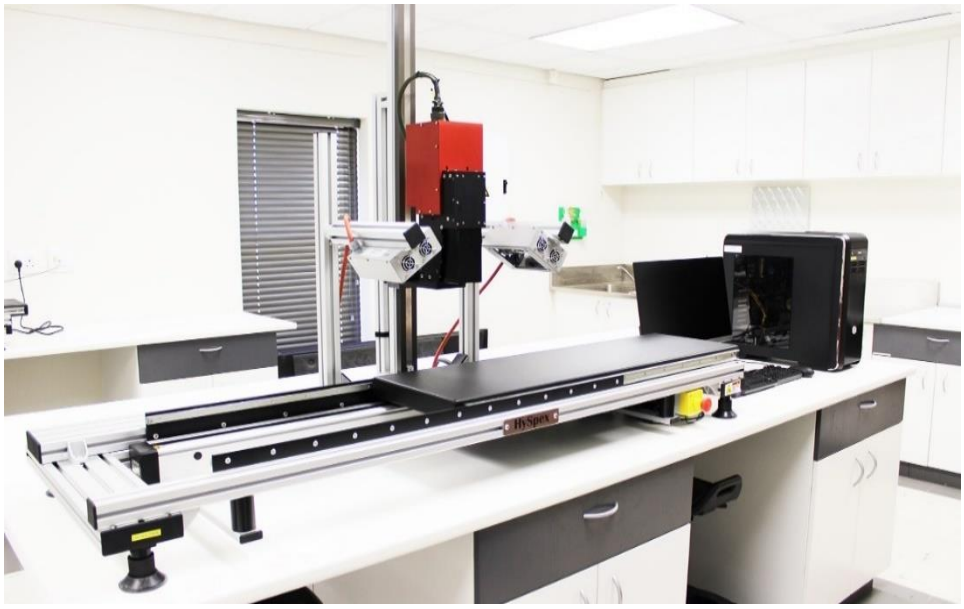


Figure 5.1 The HySpex SWIR-384 pushbroom imaging system consisting of light source, filter, camera, detector, sample stage and computer.

5.2.3 Quality assessment and chemical analysis

After hyperspectral imaging, the reference values for the HU, protein (%), lipid (%) and moisture (%) was determined for all the eggs to select a calibration and validation set for each of these egg parameters. Protein, lipid and moisture content was determined as g/100 g and expressed as a percentage (%). The HU was used to determine the freshness of each egg using Equation 3.2 (Section 3.2.2). During analysis, the egg yolks for some of the eggs ruptured, which made it difficult to measure the thick albumen height. As a result, the HU for only 408 out of the 464 egg could be calculated. The moisture, lipid and protein content of all the eggs were calculated using Equation 3**.3**, Equation 3.6 and Equation 3.7 respectively as described by the Association of Official Analytical Chemists (AOAC, 2002) explained in Section 3.2.3. The protein and lipid content were determined on a dry matter basis for each sample. Equation 5.1 was used to convert the percentage protein (crude protein - CP) and lipids calculated on a dry matter basis to the protein and lipid content of the whole liquid egg (fresh weight basis). The obtained values were expressed as gram protein or lipid per gram fresh weight. The equation was implemented since hyperspectral images were collected of the whole intact liquid egg and not on the dry matter.

Equation 5.1:

$$a = \left(b \times \frac{100}{(100-c)}\right) \times \left(\frac{(100-d)}{(100)}\right)$$

where:

a = % Protein or Lipid content of initial whole liquid egg

b = % CP or lipid of dry sample

c = % Moisture obtained by the dry sample during handling

d = % Moisture of whole liquid egg after freeze drying

5.2.4 Image correction and cleaning

Sample images obtained from the HySpex hyperspectral imaging instrument were transformed from instrumental reflectance counts to absorbance values by using Equation 5.2 (Sendin, 2017) and was automatically performed in Evince software version 2.7.11 (Prediktera AB, Umeå, Sweden).

Equation 5.2:

$$I_{\lambda,n} = -log\left[\left(\frac{S_{\lambda,n} - B_{\lambda,n}}{W_{\lambda,n} - B_{\lambda,n}}\right)\right] \times x$$

where:

n = Pixel index variable (n = 1…N) of the re-organized hypercube

$I_{\lambda,n}$ = Standardised absorbance intensity, pixel n, at wavelength λ

$S_{\lambda,n}$ = Sample image, pixel n, at wavelength λ

$B_{\lambda,n}$ = Dark reference image, pixel n, at wavelength λ

$W_{\lambda,n}$ = White reference image, pixel n, at wavelength λ

x = Total reflectance

Prior to model development, the individual images were merged using Evince software to form mosaics. Principal component analysis was conducted and the principal component plots used to remove unwanted information related to the background, differences in sample illumination and shading (Geladi *et al.*, 1989) and noise (Beebe *et al.*, 1998; Cen & He, 2007). Specular reflectance on the glossy oval shape of the egg surface, caused by the light source, as observed in Figure 5.2 was excluded, as it may contribute to noisy spectra (Wold *et al.*, 2001). The images were cleaned and the PCA was recalculated. The average spectrum was obtained for each egg and all subsequent analyses was conducted using the average spectra for the eggs.

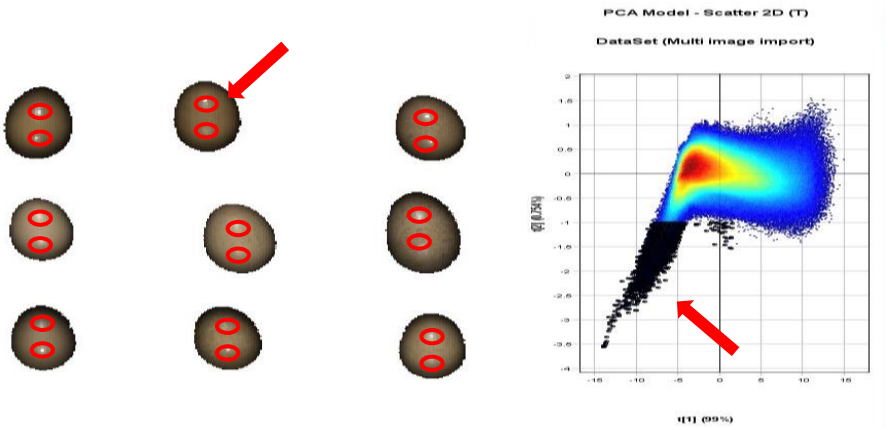


Figure 5.2 Illustration of the removal of specular reflectance due to the glossy oval shape of the egg (red circles and arrows indicated the unwanted pixels corresponding to the specular reflection in the images).

5.2.5 Principal component analysis to explore trends in the dataset

To demonstrate the application of PCA for exploring trends in the dataset two approaches were taken. First, individual images of eggs from the 21-week age group (59 in total), which were stored for different time periods were merged. A second analysis where images for eggs (79 in total) collected at day 0 from different aged hens was conducted. This was done to investigate if it was possible to see differences between (i) eggs stored for different time periods (Section 5.3.2.1) and (ii) eggs laid by different age hens (Section 5.3.2.2). The data were analysed with an object-wise PCA approach by comparing the average spectra of the eggs using the Evince software.

During PCA, a two-dimensional dataset (λ; x·y) containing the information for the loadings and scores is generated (Equation 2.1). A loadings plot can be used to identify the wavelengths for each principal component, which contribute to the variation between samples and/or clusters that may be present. When selecting the number of principal components (PC) to use, it is possible that only the first PC displays the separation between the different samples (September, 2011; Bezuidenhout *et al.*, 2018).

Various preprocessing techniques were evaluated including mean-centering, standard normal variate (SNV) (Barnes *et al.*, 1989) and Savitzky-Golay 1st Derivative 2nd Polynomial 15 point window (Rinnan *et al.*, 2009). The PCA score plots and loading line plots were investigated.

5.2.6 Partial least squares regression (PLSR)

For this section, PLS models were developed, which incorporated all the samples including eggs laid by different aged hens and eggs stored for different durations to generate a dataset representing all of the variation. The procedures of imaging and sample analysis are outlined in Figure 5.3 and include sampling, image acquisition, analytical measurement of chemical parameters, image processing, reduction in wavelength range and development of PLS calibrations. Two approaches to optimize models to predict each of the parameters separately were attempted. The first approach included the comparison of models constructed with the whole egg spectra and models generated with spectra selected from a smaller region of interest (ROI). During the second approach, models that gave the best results when constructed with either the whole egg spectra or ROI spectra were selected and used for further optimization through testing a reduced wavelength region to exclude some of the noisy data at specific spectral wavebands at the beginning and end of the spectra. Details of the PLS model development are described in this section.

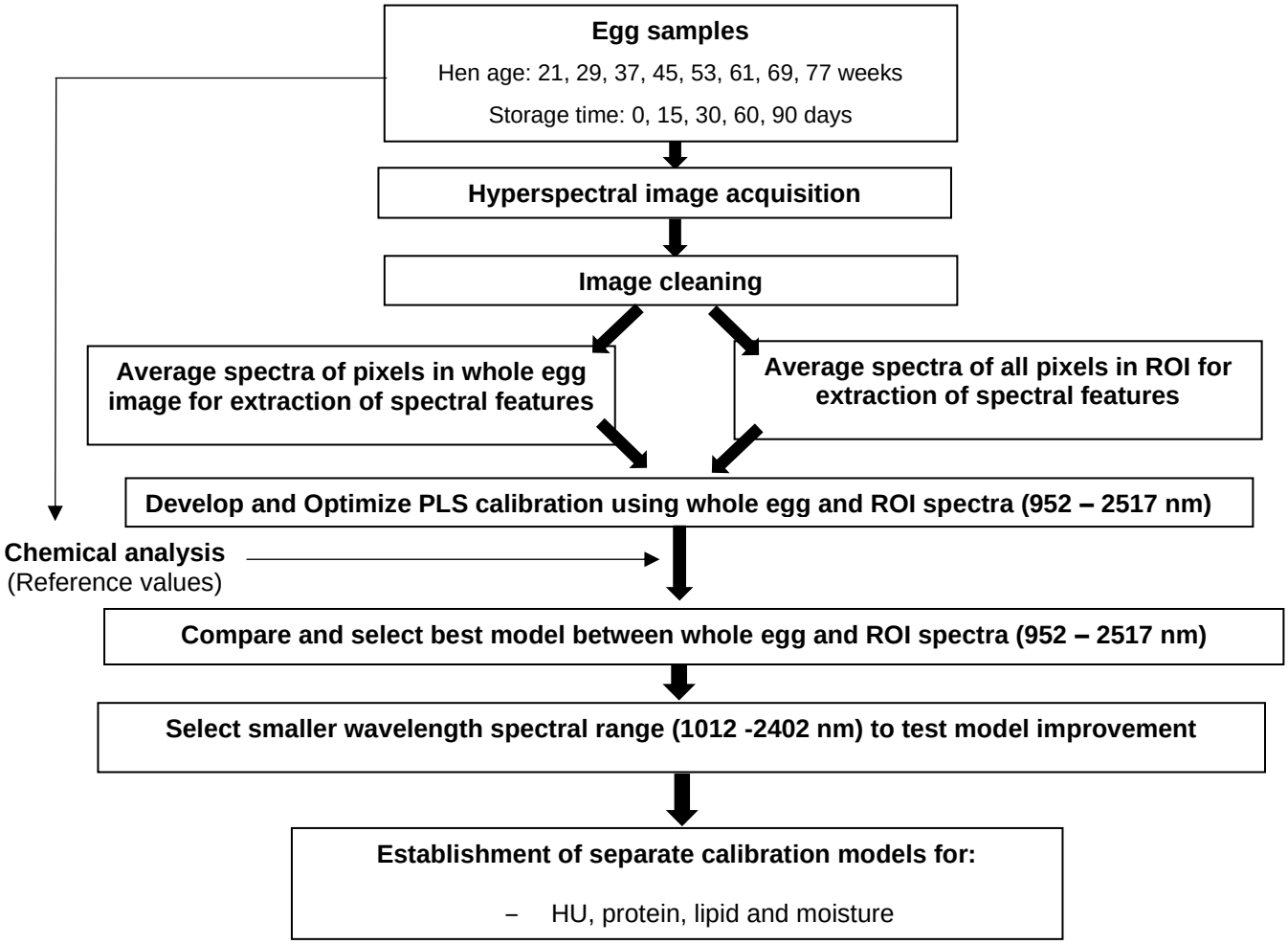


Figure 5.3 Diagram illustrating analytical approach to develop and optimize calibrations to predict quality parameters of eggs.

5.2.6.1 Data preprocessing

The average spectral data was used to develop separate PLS calibrations for each chemical parameter (HU, protein, lipids and moisture). During model development several preprocessing techniques were applied and the best number of latent variables were chosen, based on the lowest possible RMSECV value, to optimize the model parameters. Preprocessing techniques were used to increase the signal from the chemical information and reduce undesirable noise (Leonardi & Burns, 1999; Letexier & Bourennane, 2008). These preprocessing techniques included standard normal variate (SNV) (Barnes *et al.*, 1989), multiple scatter correction (MSC) (Geladi *et al.*, 1985) and lastly Savitzky-Golay 1st derivative 2nd Polynomial as well as Savitzky-Golay 2nd derivative 3rd Polynomial both with a 15 point window size (Rinnan *et al.*, 2009; Vidal & Amigo, 2012). To enhance the differences between the samples, mean centering was subsequently applied, after each individual preprocessing technique.

5.2.6.2 Whole egg image spectra and selection of ROI

Prior to preprocessing, the regions of interest (ROI) were selected for each sample. Selecting a region of interest can have a significant impact on the effectiveness of the prediction model (Barbin *et al.*, 2012). The ROI consists of a smaller number of pixels with similar spectral characteristics as the spectra of the whole egg (Zhang *et al.*, 2015). The ROI consisted of a size of 40 x 40 pixels obtained at the equatorial region of each egg as suggested by Suktanarak and Teerachaichayut, (2017). The ROI was also selected in this region to exclude the air cell, located at the blunt end of the egg, which can have an influence on the specular characteristics and thus influence the overall robustness of the model. The average spectra of the whole egg image and ROI were used as representation of the sample during subsequent analysis. The average spectra were exported in MATLAB format and imported in PLS toolbox software version 8.6.2 (Eigenvector Research Inc., Wenatchee, WA, USA).

5.2.6.3 Wavelength spectral range selection

After evaluating the predictive performance of models developed from the whole egg spectra and ROI spectra, a shorter wavelength region was selected. For the first optimization trial, the full wavelength range was used (952 - 2517 nm). Further optimization by removing the spectral wavebands from 952 to 1011 nm and 2402 to 2517 nm at the beginning and end of the spectra were implemented and analysis was performed using the 1012 to 2402 nm wavelength region. The removal of these wavelengths associated with noisy data were performed to evaluate if the models would improve. This approach is a useful technique in spectroscopic data analysis (Balabin & Smirnov, 2011) as the removal of irrelevant wavebands can increase the accuracy of prediction and improve the overall robustness of the model (Wu & He, 2006; Cheng *et al.*, 2013).

5.2.6.4 Development and evaluation of calibration model

The dependent variables were the reference values (HU, protein, lipids and moisture), whilst the spectra (wavelength range 952 – 2517 nm and 1012 – 2402 nm) were the independent variables. The samples were divided in a ratio of 70:30 to obtain a calibration and validation set. The samples used for the calibration set were chosen to represent the entire range of reference values for each respective parameter, whilst the samples for the validation set were selected to fall within the range (Lin *et al.*, 2011).

The models were constructed with the calibration set and the accuracy of the models' prediction were evaluated using the validation set. Cross validation using venetian blinds was performed during model development with 16 data splits and five samples per blind (thickness). The accuracy or performance of the models was tested using various indicators. These include the coefficient of determination (R^2) value, root mean square error of cross validation (RMSECV) and root mean square error of prediction (RMSEP). The RMSECV and RMSEP are calculated according to Equation 5.3, where the unit of measure for protein, lipid and moisture was 1g per 100g.

The R^2-value indicates the percentage variance explained by the model. The optimum conditions to construct the calibration model should give the highest possible value for R^2 and lowest possible value for RMSECV, while the number of principal components should be low. The validation set was used to evaluate the performance of each model and enable selection of the best model by evaluating the R^2 and RMSEP values. RMSEP indicates the accuracy of prediction based on data used from the validation set or an external dataset (Burger, 2006). The effectiveness of the model for routine analysis, is evaluated by using the ratio of prediction to standard deviation (RPD) value (Saeys *et al.*, 2005). The RPD value is dimensionless and can be used to assign the model to different classes of effectiveness (Saeys *et al.*, 2005). If the RPD value was less than 1.5 the model was ineffective, between 1.5 and 2.0 the model can only differentiate between high and low values, between 2.0 and 2.5 the model can make approximate predictions, between 2.5 and 3.0 the model offers acceptable predictions and an RPD value above 3.0 is excellent for prediction.

Equation 5.3:

$$RMSECV\ or\ RMSEP = \sqrt{\frac{\sum_{i=1}^{n}(y_{model} - y_{measure})^2}{n}}$$

where:

y_{model} = Predicted values from the model (calibration or cross validation for RMSECV or RMSEP)

$y_{measure}$ = Measured values obtained from analytical or chemical measurements (HU, protein, lipids and moisture)

5.3 Results and discussion

5.3.1 Spectral analysis

The average spectra of the egg samples have similar absorption bands. The most prominent bands are seen at 1465, 1939, 2162 and 2312 nm (Figure 5.4). The 1465 nm band is related to the N-H stretch first overtone from protein and/or (-OH stretch first overtone of water as stated by Osborne *et al.* (1993), also confirmed in other studies (Uddin *et al.*, 2006; Alexandrakis *et al.*, 2012; Zhao *et al.*, 2018). The 1939 nm absorption band can be attributed to the O-H stretching and O-H deformation related to moisture (Osborne *et al.*, 1993; Manley, 2014; Bezuidenhout *et al.*, 2018). Finally, the 2162 nm band represents the amide groups present in protein and the 2312 nm band represents the C-H stretch and C-H deformation associated with lipids. Both the 2162 and 2312 nm absorption bands were consistent with those indicated by Wehling *et al.* (1988).

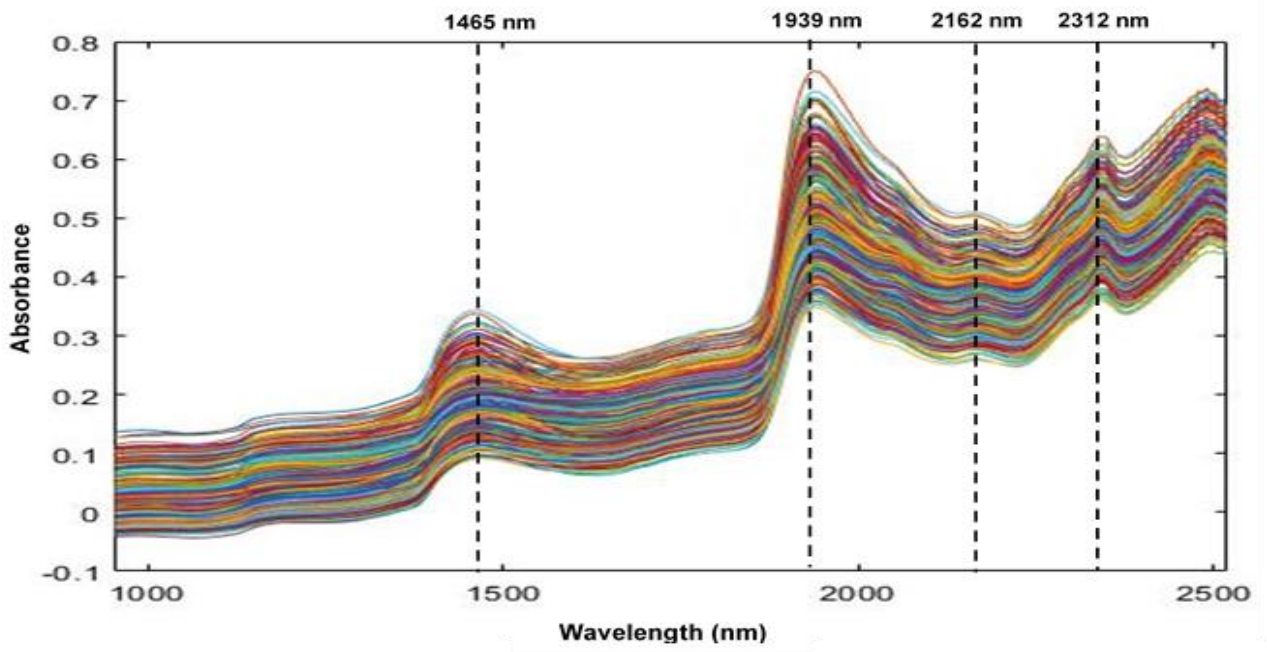


Figure 5.4 Raw spectra of all egg sample illustrating important spectral peaks.

5.3.2 Principal component analysis for exploratory analysis

Principal component analysis was applied to explore trends in the two different datasets. Firstly, PCA was applied to investigate if it is possible to distinguish between eggs laid by different aged hens. Secondly, PCA was applied to investigate if it is possible to distinguish between eggs stored for different time periods. The application of SNV preprocessing performed better than the other preprocessing techniques for identification of clusters and only these results are reported here.

5.3.2.1 Applying PCA to distinguish between eggs laid by different aged hens

Scatter (score) plots and loadings plots were used to explore the data. At least four PC's in different combinations were investigated to evaluate if separation between eggs laid by different hen age groups could be identified. Since chemical differences were observed between eggs collected from different age groups (Chapter 3), it was expected that samples would clusters based on differences in the chemical composition.

The score plot of PC1 vs. (*versus*) PC2 (Figure 5.5a) showed separation between younger hen age groups (21W, 29W and 37W) and older hen age groups (69W and 77W) along PC1, which accounted for 78.1% of the variation in the data (Figure 5.5a). Furthermore, PC2, PC3 and PC4 accounted for 17.3%, 2.7% and 0.8% of the variance in the data respectively, however no further separation or clustering was observed in other combination of PC's. For this reason, these combinations were not analyzed further.

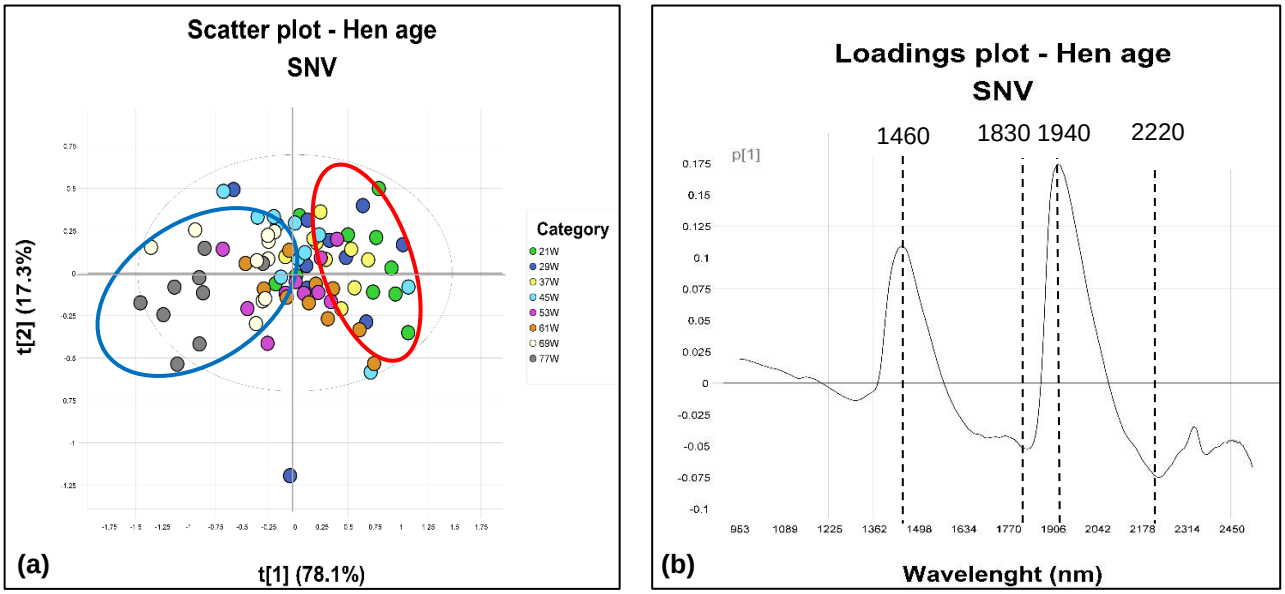


Figure 5.5 Illustration of (**a**) PCA score plot of PC1 (78.1%) vs. PC2 (17.3%) and (**b**) PCA loadings line plot for PC1 with wavebands at 1460, 1830, 1940 and 2220 nm after the application of SNV preprocessing. Different colours in the score plot correspond to the age groups of the hens (W – weeks). The red circle indicates samples from the 21, 29 and 37 week age group. The blue circle indicates samples from the 69 and 77 week age group. Clusters separate along PC1.

To evaluate the trends observed in the scatter plot, the loadings plot for PC1 (Figure 5.5b) was analyzed along with the score plot (Figure 5.5a) to identify the wavebands, which contribute most to the differences observed between the categories (hen age groups) (Bezuidenhout *et al.*, 2018). In the loadings plots (Table 5.5b), four peaks were identified which contributed to the separation between the clusters. Two bands at 1460 and 1940 nm with positive loadings and two bands at 1830 and 2220 nm with negative loadings contributed to the separation between the clusters (Table 5.5b).

These wavebands correspond to differences in the chemical composition of the eggs collected from various age groups and appeared to be related to the compounds that were measured during the proximate analysis and make up most of the content of the egg. To evaluate this, the proximate composition for the moisture, protein and lipid content was recalculated on a wet basis and differences could be observed (Table 5.2). In summary, the moisture content decreased as the hens age, whilst the protein and lipid content increased with hen age.

Table 5.2 The means ± standard deviations of proximate composition for eggs laid by hens with ages ranging from 21 to 77 weeks

Parameter		Wet Basis			
		Moisture (%)	Protein (%)	Lipids (%)	Lipid : Protein
Treatment (Hen Ages in Weeks)	**21**	76.62^{a} ± 1.55	12.26^{c} ± 1.05	8.53^{e} ± 1.12	0.69^{c}
	29	75.48^{ab} ± 1.63	12.85^{bc} ± 0.87	9.09^{de} ± 1.00	0.71^{bc}
	37	75.20^{ab} ± 1.66	12.94^{bc} ± 0.78	9.40^{cde} ± 1.26	0.72^{bc}
	45	74.14^{bc} ± 1.59	13.15^{ab} ± 0.87	9.75^{bcd} ± 1.21	0.74^{abc}
	53	73.57^{c} ± 1.46	13.10^{ab} ± 0.64	10.06^{abcd} ± 1.33	0.76^{ab}
	61	73.59^{c} ± 1.83	13.13^{ab} ± 1.08	10.23^{abc} ± 1.23	0.77^{ab}
	69	73.16^{c} ± 1.70	13.58^{ab} ± 0.85	10.79^{ab} ± 1.42	0.79^{a}
	77	73.09^{c} ± 1.79	13.80^{a} ± 0.54	11.09^{a} ± 1.12	0.80^{a}
	P value	<0.01	0.03	<0.01	0.02

($^{a, b, c, d, e}$) Means with different superscripts within the same column differ significantly (P≤0.05)

The 1940 nm spectral band contributed most to the separation between the eggs. This band is related to moisture [O-H stretch, O-H deformation combination and O-H bend second overtone] (Osborne *et al.*, 1993; Shark *et al.*, 2001; Manley, 2014). The separation implies that eggs laid by younger hen ages had a higher moisture content than eggs laid by older hen ages. The proximate analysis confirmed that eggs from younger hens had a higher moisture content, which decreased as the hens' age increased. The 1460 nm band is related to the N-H stretch first overtone from protein and/or the -OH stretch first overtone of water (Osborne *et al.*, 1993; Liu *et al.*, 2003; Zhao *et al.*, 2018). The PCA score and loadings plot indicated that eggs laid by hen ages 21, 29 and 37 weeks had a higher protein/water content than eggs laid by older hens (Figure 5.5). However, the protein content in the eggs displayed an opposite trend and decreased in eggs as the hens aged. This band may therefore correspond to the water content of the eggs, rather than the protein content.

The loadings plot of PC1 revealed two wavelengths with negative loadings. The first at 1830 nm [O-H stretch and 2 × C-O stretch] was previously associated with cellulose (Osborne *et al.*, 1993). However, eggs do not contain cellulose as it is a polysaccharide produced by plants (Preston & Cronshaw, 1958; O'Sullivan, 1997). This band might be associated with carbohydrates (Osborne *et al.*, 1993), but this result cannot be confirmed as the carbohydrate content of eggs are very low and constitute less than 1% of the egg content of a large egg (Powrie & Nakai, 1986). For this reason, the carbohydrate content was not determined in the current study. However, a study done by Yadgary *et al.* (2010) concluded that the carbohydrate content in the yolks of eggs laid by older hens (50 weeks) was higher when compared to eggs laid by younger hens (30 weeks).

The 2220 nm wavelength [C-H stretch and C=O stretch] is associated with a -CHO structure, which may be attributed to lipids (Osborne *et al.*, 1993; Jaillais *et al.*, 2007). The trend in the PCA score and loadings plot (Figure 5.5) indicated a lower lipid content in eggs laid by 21-, 29- and 37-week-old hens compared to eggs laid by 69 and 77-week-old hens. This was confirmed by Table 5.2, where eggs laid by 21, 29 and 37 weeks of age displayed a low lipid content (8.53%,

9.09% and 9.40%, respectively) and eggs laid by 69- and 77-week-old hens displayed higher lipid content (10.79% and 11.09, respectively). The increase in the lipid content with increase in hen age is due to older hens depositing more lipids in the egg yolks compared to eggs laid by younger hens, as explained in Section 3.3.3. The increased lipid content in older hens was also confirmed by Ahn *et al.* (1997).

In Table 5.5a it is noted that the rest of the hen ages (45W, 53W and 61W) are clustered in the center of PC1. This suggest that eggs laid by these age groups have intermediate moisture, protein and lipid content compared to the younger and older hen ages, which is confirmed by the results in Table 5.2.

In summary, the results above suggest that PCA could be used to identify sample clustering and by analyzing corresponding reference data, the concentration gradients responsible for the variation between eggs laid by different hen ages could be evaluated.

5.3.2.2 Applying PCA to distinguish between eggs stored for different time periods

Scatter (score) plots and loadings plots were also used to explore the data and identify patterns between the eggs stored for different time intervals. Once again, SNV preprocessing resulted in trends that could be interpreted. Four PC's in different combinations were investigated to identify separation between eggs with different storage times. PC1 accounted for 87.7% of the variance in the data and PC2 for 8.83% of the variance in the data (Figure 5.6a). Furthermore, PC3 and PC4 accounted for 1.8% and 0.7% of the variance in the data, respectively. A clear separation between samples based on the storage time was visible along PC1 (Figure 5.6a). Since no additional separation or clustering was observed in the score plots between PC2, PC3 and PC4, these were not reported. The score plot of PC1 vs. PC2 (Figure 5.6a) showed three clusters between storage intervals. The first cluster contained samples from Day 0, the second cluster contained samples from Day 15 and the third cluster contained samples from Day 45, 60 and 90, while samples from Day 30 was spread between the three clusters. This suggests that major chemical changes occur within the first 15 days of storage.

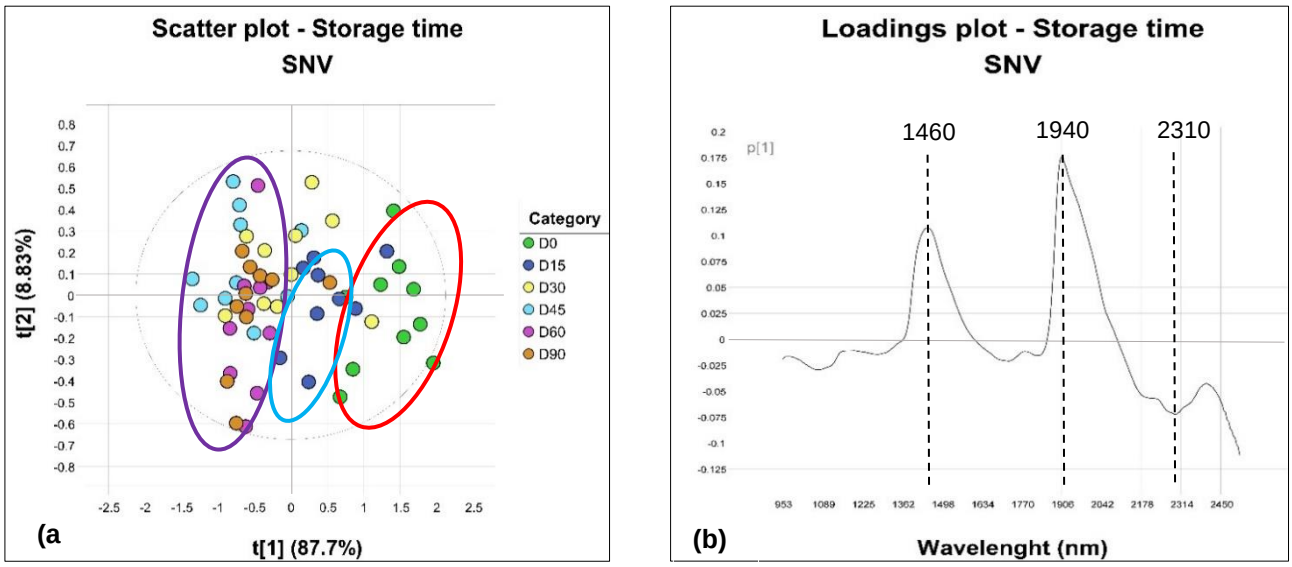


Figure 5.6 Illustration of (**a**) PCA score plot of PC1 (87.7%) vs. PC2 (8.83%) and (**b**) PCA loadings line plot for PC1 with wavebands indicated at 1460, 1940 and 2310 nm after the application of SNV preprocessing. Different colours in the score plot correspond to the storage period (D – days) of the eggs. The red circle indicates samples from the Day 0 group. The blue circle indicates samples from the Day 15 group. The purple circle indicates samples from the Day 45, 60 and 90 group. Clusters separate along PC1.

Evaluation of the loadings plot for PC1 indicated that there are two wavebands with positive loadings at 1460 and 1940 nm and one band at 2310 nm with a negative loading value. Some of the wavebands (1460 and 1940 nm) are similar to the ones identified during the analysis of eggs from the different hen age groups (Figure 5.6b). It was expected that the clusters observed would correspond to the proximate changes detected. Analysis of the results (Table 5.3), indicated a noteworthy change in the moisture, protein and lipid content within the first 15 days of storage and that the internal composition of eggs stored for different time periods changed over time. The proximate results (Table 5.3) were used, along with the results in Chapter 4 to interpret the results obtained in the PCA score plot (Figure 5.6a) and loadings plot (Figure 5.6b).

The 1940 nm spectral band once again accounted for most of the variation observed. This waveband is related to moisture [O-H stretch, O-H deformation combination and O-H bend second overtone] (Osborne *et al.*, 1993; Shark *et al.*, 2001; Manley, 2014). The 1460 nm band is related to the N-H stretch first overtone from protein and/or the -OH stretch first overtone of water (Osborne *et al.*, 1993; Liu *et al.*, 2003; Zhao *et al.*, 2018). The band at 2310 nm [C-H stretch and C-H deformation] is associated with lipids (Wehling *et al.*, 1988; Osborne *et al.*, 1993). The results indicate that the separation between the clusters in Figure 5.6a can be explained by differences in their moisture content. Eggs from day 0 had a higher moisture content, which decreased as the egg's storage period became longer (Day 45, 60 and 90). The results from the proximate analysis indicated that day 45, 60 and 90 showed no significant differences in terms of moisture content (Table 5.3) and this was confirmed by the PCA score plot (Figure 5.6a) where eggs from these storage groups clustered together.

Table 5.3 The means ± standard deviations of proximate composition for eggs laid by hens at 21 weeks of age stored for 90 days

Parameter		Moisture (%)	Wet Basis		
			Protein (%)	Lipids (%)	Lipid:Protein
Treatment (Storage Time in Days)	0	76.62^{a} ± 1.55	12.26^{e} ± 1.05	8.53^{c} ± 1.12	0.69
	15	74.77^{b} ± 1.63	13.14^{d} ± 1.08	9.41^{b} ± 0.46	0.71
	30	74.03^{bc} ± 1.19	13.88^{cd} ± 0.52	9.81^{b} ± 0.68	0.71
	45	73.04^{cd} ± 1.07	14.04^{bc} ± 0.61	10.19^{ab} ± 0.85	0.72
	60	72.48^{d} ± 1.55	14.76^{ab} ± 0.62	10.63^{a} ± 1.14	0.72
	90	72.26^{d} ± 1.41	14.91^{a} ± 0.80	10.86^{a} ± 0.78	0.72
	P value	<0.01	<0.01	<0.01	0.83

($^{a, b, c, d, e}$) Means with different superscripts within the same column differ significantly (P≤0.05)

The results from the chemical analysis (Table 5.3) indicated that the moisture content was highest in eggs stored for 0 days (76.62%), followed by 15 days (74.77%) and eggs stored for 45, 60 and 90 days had the lowest moisture content (73.04%, 72.48% and 72.26% respectively). The decrease in the moisture content can be attributed to the movement of moisture from the albumen, through the eggshell pores to the surrounding environment, as explained in Section 4.3.3. The PCA score and loadings plot also indicated that eggs stored for longer periods of time showed a lower protein and/or moisture content. The reduced protein content is not confirmed by the proximate results in Table 5.3, which indicates an increase in protein content. This waveband might therefore correspond to the decrease in moisture content.

The waveband 2310 nm has negative loadings and the trend in the PCA score plot (Figure 5.6a) suggests that this substance is higher in eggs stored for longer periods. Analysis of the proximate results indicated that the lipid content of eggs stored for 0 days was the lowest (8.53%), followed by 15 days (9.41%) and eggs stored for 45, 60 and 90 days displayed the highest lipid content (10.19%, 10.63% and 10.86%, respectively). Ahn *et al.* (1997) stated that an increase in storage time can result in the loss of moisture, which could lead to the increased lipid (solid) percentage (higher moisture to lipid ratio). This would explain the increase in lipid concentration observed in Table 5.3 and the increase displayed by the PCA analysis (Figure 5.6) in the current study. Eggs from day 45, 60 and 90 clustered together in the PCA score plot (Figure 5.6a), suggesting little differences in lipid content. This trend is confirmed by the results obtained in Table 5.3, displaying no significant difference in the lipid content between these three storage intervals.

The results above suggest that PCA could be used to identify sample clustering and by analyzing corresponding reference data, the concentration gradients of chemicals responsible for the variation between eggs with different storage durations could be visualized using PCA plots.

5.3.3 Development of the PLS models for various chemical parameters using whole egg and ROI spectra

The reference values for each chemical parameter, which was used to generate the calibration and validation set are summarized in Table 5.4. Samples were carefully selected to generate each dataset. This is important because, if for example one or more values in the validation set fall outside of the calibration range, the model will not be able to predict these samples accurately. The calibration and the validation sets' standard deviations (SD) were similar for each respective parameter (Table 5.4). The values for the HU and moisture spanned a broad range, whilst the values for the protein and lipid content spanned a narrower range (Table 5.4). A wider range of HU and moisture values, might be more favourable for calibration development (Ripoche & Guillard, 2001).

Table 5.4 Components of HU, protein, lipid and moisture's calibration and validation set

		N	Range	Mean	SD
HU	Calibration set	290	10.18 - 104.05	50.79	26.00
	Validation set	118	15.06 - 103.75	50.18	27.59
Protein (%)	Calibration set	322	10.53 - 17.45	14.22	1.05
	Validation set	142	11.98 - 16.01	13.88	1.01
Lipids (%)	Calibration set	322	5.82 - 15.27	10.63	1.28
	Validation set	142	6.26 - 14.89	10.34	1.20
Moisture (%)	Calibration set	322	66.16 - 78.91	72.06	2.20
	Validation set	142	68.06 - 77.84	71.95	2.16

N = Number of samples.
SD = Standard deviation.

5.3.3.1 Testing the accuracy of the models using whole egg spectra and ROI spectra

The average whole egg spectra of samples were used to develop the calibration model. To optimize the model parameters, cross validation and various spectral pretreatments were applied to select the optimal number of PC's for each of the models for HU, protein, lipid and moisture content (Table 5.5). Model parameters (R^2 and RMSECV) for each pretreatment method are summarize in Table 5.5 for the whole egg spectra within the 952 – 2517 nm wavelength range. The unit of measure for RMSECV and RMSEP, in terms of the protein, lipid and moisture model is expressed as gram per 100 gram (g/100g).

Table 5.5 Results for various spectral pretreatment techniques to develop models for the prediction of egg quality parameters in the calibration set of the whole egg spectra in the wavelength range 952 – 2517 nm by using cross validation

Model	Pretreatment	N	F	Cross Validation	
				R^2	RMSECV
HU	Mean Centering (MC)	290	5	0.45	18.99
	SNV and MC	290	7	0.54	17.39
	MSC and MC	290	5	0.36	20.55
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	290	8	0.59	16.47
	Savitzky-Golay 2nd Derivative 3rd Polynomial and MC	**290**	**8**	**0.60**	**16.30**
Protein (g/100g)	Mean Centering (MC)	322	10	0.47	3.15
	SNV and MC	322	10	0.48	3.13
	MSC and MC	322	11	0.4	3.37
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	322	10	0.52	3.02
	Savitzky-Golay 2nd Derivative, 3rd Polynomial and MC	**322**	**10**	**0.53**	**2.99**
Lipid (g/100g)	Mean Centering (MC)	322	11	0.45	3.95
	SNV and MC	322	8	0.42	4.04
	MSC and MC	322	12	0.39	4.16
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	322	10	0.46	3.89
	Savitzky-Golay, 2nd Derivative, 3rd Polynomial and MC	**322**	**11**	**0.47**	**3.86**
Moisture (g/100g)	Mean Centering (MC)	322	12	0.52	7.26
	SNV and MC	322	10	0.48	7.55
	MSC and MC	322	12	0.45	7.84
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	**322**	**12**	**0.53**	**7.24**
	Savitzky-Golay 2nd Derivative, 3rd Polynomial and MC	322	10	0.51	7.37

N = Number of samples; F = Number of principal components used.
SNV = Standard normal variate; MSC = Multiple scatter correction.
R^2 = Coefficient of determination; RMSECV = Root mean square error of cross validation.

From Table 5.5, it can be concluded that Savitzky-Golay 2nd Derivative, 3rd Polynomial, 15 point (pt) window size (SG2D3P) was the preferred method for developing the calibration models for the HU, protein and lipid content. The Savitzky-Golay 2nd Derivative, 3rd Polynomial preprocessing technique with eight principal components gave the best results (R^2 = 0.60 and RMSECV = 16.30) for establishing the HU calibration model. For the protein model, Savitzky-Golay 2nd Derivative, 3rd Polynomial with ten principal components resulted in the best model (R^2 = 0.53 and RMSECV = 2.99 g/100g). The Savitzky-Golay 2nd Derivative, 3rd Polynomial pretreatment with 11 principal components was also optimal for developing the lipid model (R^2 = 0.47 and RMSECV = 3.86 g/100g). Finally, Savitzky-Golay 1st Derivative, 2nd Polynomial with 12 principal components gave the best results for the moisture model (R^2 = 0.53 and RMSECV = 7.24 g/100g).

After each model parameter was optimized, using the whole egg spectra, the models were used to test the accuracy of prediction (or model validation). Model validation is one of the most important tasks in multivariate data analysis. It aims to assess the prediction accuracy of models by using the validation set (Li *et al.*, 2016). Only the model parameters that gave the best results in Table 5.5 were applied and the results were recorded in Table 5.6. The precision and stability of the model can be evaluated using the coefficient of determination (R^2), root mean square error of prediction (RMSEP) and ratio of deviation to prediction (SD:RMSEP or RPD value) after application to the validation set (Cheng *et al.*, 2013).

Table 5.6 PLSR model using whole egg spectra for predicting egg chemical parameters in the calibration and validation set (952 – 2517 nm)

Model	Pretreatment	Calibration Set				Validation Set				
		N	F	R^2	RMSECV	N	F	R^2	RMSEP	RPD
HU	SG2D3P	290	8	0.60	16.30	118	8	0.69	15.88	1.78
Protein (g/100g)	SG2D3P	322	10	0.53	2.99	142	10	0.59	2.51	1.56
Lipid (g/100g)	SG2D3P	322	11	0.47	3.86	142	11	0.63	3.01	1.64
Moisture (g/100g)	SG1D2P	322	12	0.53	7.24	142	12	0.66	6.20	1.72

N = Number of samples; F = Number of principal components used for PLSR; R^2 = Coefficient of determination.
RMSECV = Root mean square error of cross validation; RMSEP = Root mean square error of prediction.
RPD = Ratio of SD to RMSEP.
SG1D2P = Savitzky-Golay 1st Derivative, 2nd Polynomial.
SG2D3P = Savitzky-Golay 2nd Derivative, 3rd Polynomial.

The HU model in Table 5.6 showed promise with eight principal components and a R^2-value of 0.69, indicating that the model worked well for predicting the test dataset. However, when the RPD was calculated, a value of 1.78 was obtained implying the model can only be used to distinguish between high and low HU values (Nicolaï *et al.*, 2007). This suggests that the model would not work well to accurately predict HU values during routine analysis. Similar results were obtained by Giunchi *et al.* (2008). They scanned whole organic eggs and when the HU model was constructed, a prediction R^2-value of 0.68 and an RPD-value of 1.8 was obtained. During their study, they used FT-NIR spectroscopy and recorded two spectral measurements (at the equatorial and air cell region) for each egg using a probe. The two measurements were average and used to carry out a PLS regression in the 1345 to 1640 nm spectral range. Their HU ranged from 83.8 to 43.7 after 16 days of storage. The study done by Giunchi *et al.* (2008) only used samples, which were kept for different storage times and all eggs were laid by 50-week-old hens.

As for the other model parameters, the protein calibration model was evaluated on the prediction set, and resulted in a R^2-value of 0.59, a RMSEP-value of 2.51 g/100g and an RPD value of 1.56. When the lipid model was applied to the prediction set, a R^2-value of 0.63, a RMSEP value of 3.01 g/100g and an RPD value of 1.64 was obtained. Application of the moisture model resulted in a R^2-value of 0.66, a RMSEP value of 6.20 g/100g and an RPD value of 1.72. From the four models, HU gave the best prediction accuracy (RPD = 1.78) and protein gave the least favorable results (RPD = 1.56). According to Saeys *et al.* (2005) models constructed with a RPD value between 1.5 and 2.0 can only be used to distinguish between samples with high and low values of that specific constituent (i.e. HU, protein, lipid and moisture), which is the case for all four models (Table 5.6).

Following the model development using the whole egg spectra, the same procedure was applied using the ROI spectra. Various preprocessing techniques were applied using the ROI spectra to optimize the HU, protein, lipid and moisture models, and the results are summarized in Table 5.7.

Table 5.7 Results for various spectral pretreatment techniques for the prediction of egg quality parameters in the calibration set of the ROI in the wavelength range of 952 – 2517 nm after applying cross validation

Model	Pretreatment	N	F	Cross Validation	
				R^2	RMSECV
HU	Mean Centering (MC)	290	6	0.37	20.39
	SNV and MC	290	8	0.45	19.02
	MSC and MC	290	9	0.44	19.12
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	**290**	**10**	**0.48**	**18.56**
	Savitzky-Golay 2nd Derivative 3rd Polynomial and MC	290	12	0.45	19.09
Protein (g/100g)	Mean Centering (MC)	322	10	0.33	3.57
	SNV and MC	**322**	**10**	**0.35**	**3.51**
	MSC and MC	322	12	0.33	3.28
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	322	10	0.34	3.54
	Savitzky-Golay 2nd Derivative, 3rd Polynomial and MC	322	12	0.29	3.72
Lipids (g/100g)	Mean Centering (MC)	322	10	0.32	4.41
	SNV and MC	322	10	0.33	4.36
	MSC and MC	322	11	0.31	4.45
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	**322**	**11**	**0.34**	**4.35**
	Savitzky-Golay, 2nd Derivative, 3rd Polynomial and MC	322	10	0.24	4.69
Moisture (g/100g)	Mean Centering (MC)	322	10	0.33	8.62
	SNV and MC	322	10	0.34	8.55
	MSC and MC	322	12	0.35	8.55
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	**322**	**12**	**0.37**	**8.37**
	Savitzky-Golay 2nd Derivative, 3rd Polynomial and MC	322	12	0.28	9.05

N = Number of samples; F = Number of principal components used
SNV = Standard normal variate; MSC = Multiple scatter correction
R^2 = Coefficient of determination; RMSECV = Root mean square error of cross validation

Table 5.7 indicated that Savitzky-Golay 1st Derivative 2nd Polynomial (SG1D2P) pretreatment worked best for developing models for the HU, lipids and moisture content. Savitzky-Golay 1st Derivative 2nd Polynomial with ten principal components (R^2 = 0.48 and RMSECV = 18.65) worked best for HU. For the protein content, SNV with ten principal components (R^2 = 0.35 and RMSECV = 3.51 g/100g) gave the best results. The lipids model showed the most promise with Savitzky-Golay 1st Derivative 2nd Polynomial with 11 principal components (R^2 = 0.34 and RMSECV = 4.35 g/100g). The moisture model showed more favorable results when Savitzky-Golay 1st Derivative 2nd Polynomial with 12 principal components was used as the pretreatment method (R^2 = 0.37 and RMSECV = 8.37 g/100g).

After the model conditions were optimized for each chemical parameter using the ROI egg spectra, the models were used to test the accuracy of prediction. Only the model parameters that gave the best results in Table 5.7 were applied and the results recorded in Table 5.8. The coefficient of determination (R^2), root mean square error of prediction (RMSEP) and RPD value were once again used to indicate the prediction accuracy of each model.

Table 5.8 Summary of PLSR model conditions for developing and predicting egg chemical parameters using the ROI egg spectra in the calibration and validation set (952 – 2517 nm)

Model	Pretreatment	Calibration Set				Validation Set				
		N	F	R^2	RMSECV	N	F	R^2	RMSEP	RPD
HU	SG1D2P	290	10	0.48	18.65	118	10	0.53	19.42	1.45
Protein (g/100g)	SNV	322	10	0.35	3.51	142	10	0.46	2.89	1.35
Lipid (g/100g)	SG1D2P	322	11	0.34	4.35	142	11	0.50	3.49	1.42
Moisture (g/100g)	SG1D2P	322	12	0.37	8.37	142	12	0.49	7.55	1.41

N = Number of samples; F = Number of principal components used for PLSR.
R^2 = Coefficient of determination.
RMSECV = Root mean square error of cross validation; RMSEP = Root mean square error of prediction.
RPD = Ratio SD to RMSEP; SNV = Standard normal variate.
SG1D2P = Savitzky-Golay 1st Derivative, 2nd Polynomial.

Application of the HU model to the validation set resulted in a R^2-value of 0.53, a RMSEP value of 19.42 and an RPD value of 1.45 (Table 5.8). Selection of the ROI to develop the model for this parameter, did not improve the model in comparison to using the whole egg spectra. This result is in contrast to the findings of Suktanarak and Teerachaichayut (2017). During their analysis, selection of a ROI improved the model, which had a R^2-value of 0.85 and an RPD value of 3.07 when using SNV preprocessing with six principal components. However, the difference in HU prediction accuracies of their study and the current one may be related to the wavelength selection and the ROI size. Suktanarak and Teerachaichayut (2017) used a shorter wavelength region of 900 - 1700 nm and a larger ROI size of 50 × 90 pixels while in the current study a wavelength range of 952 - 2517 nm with a ROI size of 40 × 40 pixels was used. It is possible that the use of a larger wavelength range and a smaller ROI in the current study resulted in a less accurate model. A larger wavelength range can include irrelevant noise or unwanted information, which can contribute to less accurate models (Ariana *et al.*, 2006; Nakariyakul & Casasent, 2008). Selection of a smaller ROI may result in excluding important information about the sample (O'Rourke *et al.*, 2011).

Application of the protein model (Table 5.8) resulted in approximately the same results (R^2 = 0.46; RMSEP = 2.89 g/100g and RPD = 1.35) when compared to the lipid and moisture model. However, the RPD of the protein model indicated the lowest prediction accuracy. When the lipid prediction model was tested a R^2 = 0.50, RMSEP = 3.49 g/100g and RPD of 1.42 was obtained. The moisture model resulted in R^2-value of 0.49, a RMSEP value of 7.55 g/100g and an RPD value of 1.41. All the models in Table 5.8 had R^2-values below 0.55, relatively high RMSEP values and low RPD values (in comparison with Table 5.6) for the prediction set, indicating poor predictive performance (Iqbal *et al.*, 2013). Since all the models constructed from the ROI spectra displayed RPD values less than 1.5, they did not meet the criteria for routine quality inspection (Saeys *et al.*, 2005).

When comparing the prediction accuracy of each of the models developed with the whole egg spectra vs the ROI, the results indicated that the HU model constructed from the whole egg spectra (R^2 = 0.69; RMSEP = 15.88; RPD = 1.78) was better compared to the ROI spectra model (R^2=0.53; RMSEP = 19.42; RPD = 1.45). For the protein model the whole egg spectral model (R^2 = 0.59, RMSEP g/100g = 2.51; RPD = 1.56; (Table 5.6) performed better than the ROI model (R^2 = 0.46;

RMSEP = 2.89 g/100g; RPD = 1.35) (Table 5.8). Similar results were observed for the lipid model with the whole egg model (R^2 = 0.63; RMSEP = 3.01 g/100g; RPD = 1.64) (Table 5.6) performing better than the ROI model (R^2 = 0.50; RMSEP = 3 .49 g/100g; RPD = 1.42) (Table 5.8). The whole egg moisture model also showed higher accuracy (R^2 = 0.66; RMSEP = 6.20 g/100g; RPD = 1.72) in Table 5.6, compared to the ROI model (R^2 = 0.49; RMSEP = 7.55 g/100g; RPD = 1.41), as seen in Table 5.8.

Overall, the models constructed using the whole egg spectra (Table 5.6) gave better results compared to the PLS models built with the ROI spectra (Table 5.8). It is not exactly clear why models constructed with the whole egg spectra gave more favourable results. It might be related to the size and position of the ROI selected, which might have been too small resulting in the exclusion of pixels with relevant information about the samples (O'Rourke *et al.*, 2011). Since the ROI was selected in the center of the egg and the egg content is not homogenized it is possible that more information related to the yolk (containing most of the lipid content), which is positioned in the center of the egg was captured. Selection of ROI may contain less relevant information from the albumen (containing most of the protein content) (Powrie & Nakai, 1986). It is possible that the position of the ROI was not a good representation of the macro-scale properties of the sample (O'Rourke *et al.*, 2011). The predictive ability of the HU model suffered the most when using the ROI spectra to develop the model. For future studies it is advised to increase the size of the ROI and/or selecting more than one ROI, for example at the sharp and blunt end as well as at the equatorial region of the egg and calculating the average spectra of the three measurements. This might be a better representation of the egg sample.

Consequently, only models constructed with the whole egg spectra were used to improve the prediction accuracy. One method to achieve higher prediction accuracy is through exclusion of noisy regions and selection of a wavelength range containing the most important wavebands (Geladi & Kowalski, 1986; Wu *et al.*, 2008).

5.3.3.2 Testing the impact of selection of a shorter wavelength range (1012 - 2402 nm) on improving the models

The wavelengths at the beginning and end of the range (Figure 5.4) may contain noise, which can have an negative influence on model development and prediction efficiency (Plaza *et al.*, 2005; Li *et al.*, 2014). Removal of these wavebands may improve the model. In this section the goal was to use the whole egg spectra and investigate if exclusion of the spectral regions from 952 to 1011 nm and 2403 to 2517 nm can improve the models. The wavelength range from 1012 to 2402 nm was used and the pretreatment methods described in Section 5.3.3 were applied to optimize the models using cross validation. The results for the new models are displayed in Table 5.9.

Table 5.9 Results for various spectral pretreatment techniques for developing models to predict different egg quality parameters using whole egg spectra in the1012 – 2402 nm wavelength range

Model	Pretreatment	N	F	Cross Validation	
				R^2	RMSECV
HU	Mean Centering (MC)	290	5	0.41	19.532
	SNV and MC	290	7	0.51	17.89
	MSC and MC	290	5	0.34	20.90
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	290	8	0.60	16.14
	Savitzky-Golay 2nd Derivative 3rd Polynomial and MC	**290**	**8**	**0.63**	**15.58**
Protein (g/100g)	Mean Centering (MC)	322	10	0.49	3.09
	SNV and MC	322	11	0.51	3.05
	MSC and MC	322	11	0.44	3.27
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	**322**	**11**	**0.55**	**2.89**
	Savitzky-Golay 2nd Derivative, 3rd Polynomial and MC	322	8	0.54	2.91
Lipids (g/100g)	Mean Centering (MC)	322	10	0.46	3.89
	SNV and MC	322	10	0.44	3.99
	MSC and MC	322	11	0.36	4.27
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	**322**	**10**	**0.50**	**3.77**
	Savitzky-Golay, 2nd Derivative, 3rd Polynomial and MC	322	8	0.49	3.79
Moisture (g/100g)	Mean Centering (MC)	322	12	0.53	7.17
	SNV and MC	322	12	0.52	7.3
	MSC and MC	322	12	0.43	7.93
	Savitzky-Golay 1st Derivative 2nd Polynomial and MC	**322**	**11**	**0.55**	**7.07**
	Savitzky-Golay 2nd Derivative, 3rd Polynomial and MC	322	8	0.52	7.26

N = Number of samples; F = Number of principal components used.
SNV = Standard normal variate; MSC = Multiple scatter correction.
R^2 = Coefficient of determination; RMSECV = Root mean square error of cross validation.

The best model for HU was obtained using Savitzky-Golay 2nd Derivative, 3rd Polynomial using eight principal components (R^2-value of 0.63 and a RMSECV value of 15.58) (Table 5.9). For the protein model, Savitzky-Golay 1st Derivative 2nd Polynomial with 11 principal components gave the best results (R^2 = 0.55 and RMSECV = 2.89 g/100g). For the lipid model, Savitzky-Golay 1st Derivative 2nd Polynomial pretreatment with ten principal components resulted in the best outcome (R^2 = 0.50 and RMSECV = 3.77 g/100g). Finally, the moisture calibration was optimal when applying Savitzky-Golay 1st Derivative 2nd Polynomial (F = 11) with a R^2-value of 0.55 and a RMSECV value of 7.07 g/100g. The model conditions that gave the best result for each of the parameters (Table 5.9) were applied to the validation set to test the accuracy of the models and the results were recorded in Table 5.10.

Table 5.10 Summary of PLSR model conditions for developing and predicting egg chemical parameters using whole egg spectra in the calibration and validation set (1012 – 2402 nm)

Parameter	Pretreatment	Calibration Set				Validation Set				
		N	F	R^2	RMSECV	N	F	R^2	RMSEP	RPD
HU	SG2D3P	290	8	0.63	15.58	118	8	0.76	14.13	2.00
Protein (g/100g)	SG1D2P	322	11	0.55	2.89	142	11	0.64	2.35	1.66
Lipids (g/100g)	SG1D2P	322	10	0.50	3.77	142	10	0.64	2.97	1.66
Moisture (g/100g)	SG1D2P	322	11	0.55	7.07	142	11	0.68	6.04	1.76

N = Number of samples; F = Number of principal components used for PLSR; R^2 = Coefficient of determination.
RMSECV = Root mean square error of cross validation; RMSEP = Root mean square error of prediction.
RPD = Ratio SD to RMSEP.
SG1D2P = Savitzky-Golay 1st Derivative, 2nd Polynomial.
SG2D3P = Savitzky-Golay 2nd Derivative, 3rd Polynomial.

In Table 5.10, the HU model showed great promise when the SG2D3P pretreatment was implemented on the calibration data and used to predict the validation set. A prediction R^2-value of 0.76 was obtained with a RMSEP value of 14.13. An RPD value of 2.00 was obtained, which indicates that the model can be used for approximate predictions of HU values (Saeys *et al.*, 2005). Giunchi *et al.* (2008) also constructed models to predict the HU of eggs laid by organically reared hens, which were stored for only 16 days. When applied to the validation set, a R^2-value of 0.68, RMSEP value of 9.1 and a RPD value of 1.8 was obtained when a straight line subtraction preprocessing (Tripathi & Mishra, 2009) method was applied. Their results indicated less favorable prediction when compared to the current study. This might be due to the use of a shorter storage period resulting in a narrower HU range of 43.7 to 83.8 (Giunchi *et al*., 2008). In the current study a 90-day storage period was used resulting in a broader HU range (4.92 - 100.87). Keeping the eggs for longer introduced more variability, which improved the robustness of the model (Thomas & Ge, 2000; Peirs *et al.*, 2003). The less accurate model constructed by Giunchi *et al.* (2008) could also be ascribed to the use of only two spectral measurements, one collected at the equatorial region and one at the blunt end using a FT-NIR spectrometer. In the current study, the entire egg was imaged and the pixel spectra were averaged. The HU model developed using the average spectra from the image displayed a better predictive performance and might be related to its ability to capture spectral and spatial information (Xing *et al.*, 2011; Wu & Sun, 2013).

Application of the protein model to the validation set, resulted in a R^2-value of 0.64, an RMSEP value of 2.35 g/100g and an RPD of 1.66. For lipids, application of the model results in a R^2 of 0.64, RMSEP of 2.97 g/100g and RPD of 1.66. The protein and lipid model had similar prediction accuracies and their RPD values suggested that the models are only capable of distinguishing between high and low protein and lipid values, respectively (Table 5.10). No literature describing the development of models to predict the protein and lipid content of intact eggs could be found. Zhao *et al.* (2018) developed highly accurate models for predicting the protein and lipid content, using spectral measurements collected for the yolk and albumen, which were homogenized separately. Two separate protein models were developed for the albumen and the yolk, whilst the lipid model was developed using only the yolk spectra. Application of their model resulted in a validation R^2-value of 0.81 for protein measured in the yolk, 0.90 for protein measured in the albumen and 0.91 for lipids measured in the yolk (Zhao *et al*., 2018). The highly accurate models can be ascribed to using homogenized samples, which avoids negative effects caused by non-uniform samples and by collecting spectral measurements on the separated egg contents (Su *et al.*, 2014). In the current study the goal was to determine if hyperspectral imaging could be used to analyze intact egg samples, which would enable automated sorting. The results suggested that analysis of intact eggs, result in models with decreased accuracy for predicting the protein and lipid content. The high prediction accuracies obtained by Zhao *et al.* (2018) might be favourable, but can only be used if the egg is broken out of its shell and homogenized, thus leading to the destruction of the sample.

Finally, when the moisture model was tested on the prediction set, a R^2-value of 0.68, a RMSEP value of 6.04 g/100g and an RPD value of 1.76 was obtained (Table 5.10). The moisture model's RPD value indicated that it could only be used to distinguish between samples with low and high moisture content. Wehling *et al.* (1988) reported a prediction accuracy of 95% (R^2 = 0.95) for their moisture model, however this was developed using homogenized, spray-dried samples as they wanted to measure the moisture accumulated by the sample after spray-drying. The high prediction accuracy obtained by Wehling *et al.* (1988) can once again be ascribed to the analysis of homogenized samples, which improves the robustness of the model as stated before. Their study also made use of spray-dried egg samples and as a result their model cannot be used for analysis of intact fresh eggs.

When comparing prediction performance of models when excluding the noisy wavelength regions (Table 5.6 vs Table 5.10) it is clear that the reduction in the wavelength range led to an improvement in prediction for the HU model (R^2 = 0.69 vs 0.76; RMSEP = 15.88 vs 14.13; RPD = 1.78 vs 2.00). For the protein model, the model also improved (R^2 = 0.59 vs 0.64; RMSEP = 2.51 g/100g vs 2.35; RPD = 1.56 vs 1.66). The lipid model displayed little change (R^2 = 0.63 vs 0.64), RMSEP 3.01 g/100g vs 2.97 and RPD value 1.64 vs 1.66). The moisture model also showed little change (R^2 = 0.66 vs 0.68; RMSEP = 6.20 g/100g vs 6.04; RPD = 1.72 vs 1.76) when comparing Table 5.6 to Table 5.10, respectively.

In summary, the exclusion of the noisy data related to the wavebands at the beginning and end of the spectra, led to increase in the accuracy of the prediction models. However, the RPD values of the protein, lipid and moisture models constructed, indicated that these models can only distinguish between high and low proximate values and the HU model can be used to make approximate predictions (Table 5.10), as explained by Saeys *et al.* (2005). Saeys *et al.* (2005) unfortunately does not explain what can be classified as low, high and approximate prediction values. These models might not be excellent for predictions but can still be used in the industry. For example, eggs with a HU above 60 can be classified as fresh and below 60 as not fresh (Zhao *et al.*, 2010), thus the HU model constructed in the current study can be used to discriminate between eggs that are safe for consumption and those that are not. The same can be said for the protein, lipid and moisture content models. It would be possible for the industry to use these models to identify and separate eggs with high and low protein, lipid and moisture content, respectively.

The results obtained in the current study suggest that future studies are required to improve the existing constructed models. In Table 5.4 it is shown that out of the 480 eggs samples only 408 of the eggs sample's HU value could be determined due to an increase incidence of ruptured yolks. These ruptured egg yolks mainly correspond to eggs stored for longer periods (45, 60, 90 days), as concluded in Chapter 4 and these samples were generally under-represented in the dataset since several eggs broke during analysis. This might possibly explain why the HU model did not show a higher prediction accuracy. Most eggs are generally consumed within 21 days after being laid, but can be kept for up to 30 days under non-refrigerated conditions and up to 45 days under refrigerated

conditions (EFSA, 2014). Significant influence of storage on the HU and proximate parameters was already observed within the first 15 days of storage. Thus, for future studies it should be considered to use a shorter storage period (e.g. 30 days) and analysing the samples more frequently during this time or increasing the sample set over this period, which might assist with improving models. Decreasing the storage period from 90 days (used in the current study) to 30 days and constructing models based on 30 days of storage, might be more valuable to the industry as well, as most egg retailers attempts to sell eggs within 21 days after the egg has been laid (EFSA, 2014). Selecting a shorter storage period may also decrease the incidence of rupturing yolks, thus preventing a decrease in the sample size. Research can also be expanded to investigate the effect of temperature and humidity changes on egg quality and if it is possible for quantification and differentiation thereof, with the aid of hyperspectral imaging. It might also be useful for future studies, to implement a classification algorithm, known as partial least squares discriminant analysis (PLS-DA), that could potentially predict samples with higher HU values (> 60) and low HU vales (< 60) qualitatively (Gowen *et al.*, 2007). Secondly, since hen age also had an influence on egg quality, there might be value in testing whether developing models based solely on hen age can also improve the models. Since eggs for some of the hen ages, for example eggs laid by 53- and 61-week-old hens, showed little differences between them in terms of egg quality, it might also be advantageous to select broader consecutive age intervals (e.g. 16 weeks), as opposed to the eight week difference between hen ages used in the current study.

5.4 Conclusion

The use of PCA has proven to be effective for data exploration, since sample separation or clusters were observed. The largest differences between egg samples laid by different aged hens and egg samples stored for different time periods were explained by PC1. This was most likely due to differences in moisture (1940 nm) and lipid (2220 nm) content present in the samples, which was indicated by the loadings line plot of PC1. For the first analysis, PC1 separated eggs laid by younger aged hens (21, 29 and 37 weeks) from older hens (69 and 77 weeks). This suggested higher moisture content with low lipid content in younger hens compared to older hens and *vice versa*. The decrease in the moisture and the increase in the lipid content with increase in hen age was confirmed by proximate content analysis (wet basis) of eggs. The trend for protein content was not confirmed, as implementation of PCA showed a decrease in protein levels with an increase in hen age, while proximate analysis showed an increase. The 1460 nm band, which was assumed to be protein, most likely corresponds to the water content. For the second analysis, PC1 separated eggs based on storage period and three distinct clusters were formed. The first cluster contained samples stored for 0 days, the second cluster samples stored for 15 days and the final cluster consisted of eggs stored for longer periods (45, 60 and 90 days). The score and loadings plot indicated that eggs stored for a shorter period of time had a higher moisture content while the lipid content was lower compared to eggs stored for longer periods. The proximate analysis confirmed that the moisture content

decreased and the lipid content increased, but did not confirm the decrease in protein content, as indicated by the PCA analysis. The protein content in the eggs displayed an opposite trend and decreased in eggs as the hens aged. This band (1460 nm) may therefore correspond to the water content of the eggs, rather than the protein content. It would be interesting to investigate if it is possible to successfully quantify lysozyme-ovomucin complex in eggs, with the aid of hyperspectral imaging and multivariate data analysis, as this protein can play a key role in egg quality.

The results obtained from the PLS models (HU, protein, lipid and moisture) created with the NIR spectral data did show potential. Models were constructed with the whole egg image spectra and ROI spectra while applying various spectral pretreatments. Models created with the whole egg spectra were superior to those constructed with the ROI spectra. This could be ascribed to the size of the ROI, likely excluding pixels with relevant information. It is suggested that future studies should increase the ROI size and selecting more than one ROI. The spectral range was also reduced from 952 - 2517 nm to 1012 - 2402 nm to evaluate if the models improved. It could be concluded that all the models improved, which can be ascribed to the exclusion of noisy spectra responsible for less accurate models. The models might not be excellent for prediction but the RPD values indicated that the protein, lipid and moisture models could distinguish between samples with high and low protein, lipid and moisture content respectively, while the HU model was able to make approximate predictions. More studies need to be done to determine whether the models will improve if the models are solely based on either hen age or storage time. It should be considered to decrease the storage time interval and increase the frequency of sample analysis during this time as well as increasing each consecutive age interval, as some of the hen ages showed little differences between them in terms of egg quality.

By using NIR hyperspectral imaging, new insights were gained in terms of egg quality and the prediction of their chemical contents in the current study. It was possible to distinguish between eggs laid by different age hens and stored for different duration in terms of their proximate composition with the aid of PCA. The constructed models showed promise and with a bit of refinement can be used as a highly successful method to quantify the composition of eggs as well as their quality. With the increase in food demand and consumers becoming more informed, a need for rapid and non-destructive analytical methods such as hyperspectral imaging is required.

5.5 References

Ahn, D. U., Kim, S. M., & Shu, H. 1997. Effect of egg size and strain and age of hens on the solids content of chicken eggs. Poult. Sci. 76, 914–919.

Alexandrakis, D., Downey, G., & Scannell, A. G. M. 2012. Rapid non-destructive detection of spoilage of intact chicken breast muscle using near-infrared and fourier transform mid-infrared spectroscopy and multivariate statistics. food bioprocess technol. 5, 338–347.

AOAC. 2002. Association of Official Analytical Chemists (AOAC) International. fficial methods Anal. AOAC Int. 17th ed. Arlington, Virginia, USA.

Applegate, T. J., Harper, D., & Lilburn, M. S. 1998. Effect of hen production age on egg composition and embryo development in commercial pekin ducks. Poult. Sci.

Ariana, D. P., Lu, R., & Guyer, D. E. 2006. Near-infrared hyperspectral reflectance imaging for detection of bruises on pickling cucumbers. Comput. Electron. Agric. 53, 60–70.

Balabin, R. M., & Smirnov, S. V. 2011. Variable selection in near-infrared spectroscopy: Benchmarking of feature selection methods on biodiesel data. Anal. Chim. Acta 692, 63–72.

Barbin, D. F., Elmasry, G., Sun, D. W., & Allen, P. 2012. Predicting quality and sensory attributes of pork using near-infrared hyperspectral imaging. Anal. Chim. Acta 719, 30–42.

Barnes, R. J., Dhanoa, M. S., & Lister, S. J. 1989. Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra. Appl. Spectrosc. 43, 772–777.

Beebe, K. R., Pell, R. J., & Beth, M. 1998. Chemometrics: A practical guide by John Wiley & Sons: New York.

Bezuidenhout, C., Williams, P. J., & Rose, L. J. 2018. Differentiation of maize ear rot pathogens, on growth media, with near infrared hyperspectral imaging. Food Anal. Methods 12, 1556–1570.

Burger, J. E. 2006. Hyperspectral NIR image analysis.

Cen, H., & He, Y. 2007. Theory and application of near infrared reflectance spectroscopy in determination of food quality. Trends Food Sci. Technol. 18, 72–83.

Cheng, J. H., Dai, Q., Sun, D. W., Zeng, X. A., Liu, D., & Pu, H. Bin. 2013. Applications of non-destructive spectroscopic techniques for fish quality and safety evaluation and inspection. Trends Food Sci. Technol. 34, 18–31.

Department of Agricalture, F. and F. 2017. Quarterly Economic Overview of Agriculture, Forestry and Fisheries Sector. 12, 1–66.

EFSA. 2014. Scientific Opinion on the public health risks of table eggs due to deterioration and development of pathogens. EFSA J. 12, 1–147.

Eisen, E. J., Bohren, B. B., & McKean, H. E. 1962. The Haugh Unit as a Measure of Egg Albumen Quality. Poult. Sci. 77, 1461–1468.

Geladi, P., Isaksson, H., Lindqvist, L., Wold, S., & Esbensen, K. 1989. Principal component analysis of multivariate images. Chemom. Intell. Lab. Syst. 5, 209–220.

Geladi, P., & Kowalski, B. R. 1986. Partial least-squares regression: A tutorial. Elsevier Science Publishers B.V.

Geladi, P., MacDougall, D., & Martens, H. 1985. Linearization and scatter-correction for near-infrared reflectance spectra of meat. Appl. Spectrosc. 39, 491–500.

Giunchi, A., Berardinelli, A., Ragni, L., Fabbri, A., & Silaghi, F. A. 2008. Non-destructive freshness assessment of shell eggs using FT-NIR spectroscopy. J. Food Eng. 89, 142–148.

Gowen, A. A., O'Donnell, C. P., Cullen, P. J., Downey, G., & Frias, J. M. 2007. Hyperspectral imaging - an emerging process analytical tool for food quality and safety control. Trends Food Sci. Technol. 18, 590–598.

Iqbal, A., Sun, D. W., & Allen, P. 2013. Prediction of moisture, color and pH in cooked, pre-sliced turkey hams by NIR hyperspectral imaging system. J. Food Eng. 117, 42–51.

Jaillais, B., Morrin, V., & Downey, G. 2007. Image processing of outer-product matrices - A new way to classify samples. Examples using visible/NIR/MIR spectral data. Chemom. Intell. Lab. Syst. 86, 179–188.

Leonardi, L., & Burns, D. H. 1999. Quantitative multiwavelength constituent measurements using single-wavelength photon time-of-flight correction. Appl. Spectrosc.

Letexier, D., & Bourennane, S. 2008. Noise removal from hyperspectral images by multidimensional filtering. IEEE Trans. Geosci. Remote Sens. 46, 2061–2069.

Li, J. L., Sun, D. W., & Cheng, J. H. 2016. Recent advances in nondestructive analytical techniques for determining the total soluble solids in fruits: A review. Compr. Rev. Food Sci. Food Saf. 15, 897–911.

Li, J., Zhang, H., Huang, Y., & Zhang, L. 2014. Hyperspectral image classification by nonlocal joint collaborative representation with a locally adaptive dictionary. IEEE Trans. Geosci. Remote Sens. 52, 3707–3719.

Lin, H., Zhao, J., Sun, L., Chen, Q., & Zhou, F. 2011. Freshness measurement of eggs using near infrared (NIR) spectroscopy and multivariate data analysis. Innov. Food Sci. Emerg. Technol.

Liu, Y., Barton, F. E., Lyon, B. G., & Chen, Y. R. 2003. Variations of large spectral sets; two-dimensional correlation analysis of loadings spectra of principal component analysis. J. Near Infrared Spectrosc. 11, 457–466.

Manley, M. 2014. Near-infrared spectroscopy and hyperspectral imaging: Non-destructive analysis of biological materials. Chem. Soc. Rev. 43, 8200–8214.

Nakariyakul, S., & Casasent, D. P. 2008. Hyperspectral waveband selection for contaminant detection on poultry carcasses. Opt. Eng. 47,.

Nicolaï, B. M., Beullens, K., Bobelyn, E., Peirs, A., Saeys, W., Theron, K. I., & Lammertyn, J. 2007. Nondestructive measurement of fruit and vegetable quality by means of NIR spectroscopy: A review. Postharvest Biol. Technol. 46, 99–118.

O'Rourke, S. M., Argentati, I., & Holden, N. M. 2011. The effect of region of interest size on model calibration for soil organic carbon prediction from hyperspectral images of prepared soils. J. Near Infrared Spectrosc. 19, 161–170.

O'Sullivan, A. C. 1997. Cellulose: The structure slowly unravels. Cellulose 4, 173–207.

Osborne, B. G., Fearn, T., & Hindle, P. H. 1993. Practical NIR spectroscopy with applications in food and beverage analysis. Harlow, England: Longman Scientific & Technical.

Peirs, A., Tirry, J., Verlinden, B., Darius, P., & Nicolaï, B. M. 2003. Effect of biological variability on the robustness of NIR models for soluble solids content of apples. Postharvest Biol. Technol. 28, 269–280.

Plaza, A., Martínez, P., Plaza, J., & Pérez, R. 2005. Dimensionality reduction and classification of hyperspectral image data using sequences of extended morphological transformations. IEEE

Trans. Geosci. Remote Sens. 43, 466–479.

Powrie, W. D., & Nakai, S. 1986. The chemistry of eggs and egg products. egg science and technology, 97–139.

Preston, R. D., & Cronshaw, J. 1958. Constitution of the fibrillar and non-fibrillar components of the walls of Valonia ventricosa. Nature 181, 248–250.

Rinnan, A. ˚, Van Den Berg, F., & Engelsen, S. B. 2009. Review of the most common preprocessing techniques for near-infrared spectra. Trends Anal. Chem. 28, 1201–1222.

Ripoche, A., & Guillard, A. S. 2001. Determination of fatty acid composition of pork fat by Fourier transform infrared spectroscopy. Meat Sci. 58, 299–304.

Saeys, W., Mouazen, A. M., & Ramon, H. 2005. Potential for onsite and online analysis of pig manure using visible and near infrared reflectance spectroscopy. Biosyst. Eng. 91, 393–402.

SAPA. 2017. Key Market Signals in the Egg Industry For the fourth quarter of 2017. , 1–29.

Sendin, K. 2017. Characterisation of whole white maize kernels using spectral imaging.

September, D. J. F. 2011. Detection and quantification of spice adulteration by near infrared hyperspectral imaging. Thesis (MSc Food Sc)--University Stellenbosch, 1–113.

Shark, J. S., Workman, J. J. J., & Westerhaus, M. O. 2001. Application of NIR spectroscopy to agricultural products. Handbook of Near-Infrared Analysis, 419–474.

Su, H., Sha, K., Zhang, L., Zhang, Q., Xu, Y., Zhang, R., Li, H., & Sun, B. 2014. Development of near infrared reflectance spectroscopy to predict chemical composition with a wide range of variability in beef. Meat Sci. 98, 110–114.

Suktanarak, S., & Teerachaichayut, S. 2017. Non-destructive quality assessment of hens' eggs using hyperspectral images. J. Food Eng. 215, 97–103.

Thomas, E. V., & Ge, N. 2000. Development of robust multivariate calibration models. Technometrics 42, 168–177.

Tripathi, S., & Mishra, H. N. 2009. A rapid FT-NIR method for estimation of aflatoxin B1 in red chili powder. Food Control 20, 840–846.

Uddin, M., Okazaki, E., Fukushima, H., Turza, S., Yumiko, Y., & Fukuda, Y. 2006. Analytical, Nutritional and Clinical Methods Nondestructive determination of water and protein in surimi by near-infrared spectroscopy. 96, 491–495.

Vidal, M., & Amigo, J. M. 2012. Preprocessing of hyperspectral images. Essential steps before image analysis. Chemom. Intell. Lab. Syst. 117, 138–148.

Wehling, R. L., Pierce, M. M., & Froning, G. W. 1988. Determination of moisture, fat and protein in spray-dried whole egg by near infrared reflectance spectroscopy. J. Food Sci. 53, 1988.

Weindl, I., Lotze-Campen, H., Popp, A., Müller, C., Havlík, P., Herrero, M., Schmitz, C., & Rolinski, S. 2015. Livestock in a changing climate: Production system transitions as an adaptation strategy for agriculture. Environ. Res. Lett. 10, 094021.

Wold, S., Sjostrom, M., & Eriksson, L. 2001. PLS-regression: a basic tool of chemometrics. Chemometrics and Intelligent Laboratory Systems. Chemom. Intell. Lab. Syst. 58, 109–130.

Wu, D., & He, Y. 2006. Vis-NIR spectroscopy for non-destructive classification of juicy peach, IEEE International Conference on Mechatronics and Automation, ICMA 2006, 84–88.

Wu, D., He, Y., & Feng, S. 2008. Short-wave near-infrared spectroscopy analysis of major compounds in milk powder and wavelength assignment. Anal. Chim. Acta 610, 232–242.

Wu, D., & Sun, D. W. 2013. Advanced applications of hyperspectral imaging technology for food quality and safety analysis and assessment: A review - Part I: Fundamentals. Innov. Food Sci. Emerg. Technol. 19, 1–14.

Xing, J., Symons, S., Hatcher, D., & Shahin, M. 2011. Comparison of short-wavelength infrared (SWIR) hyperspectral imaging system with an FT-NIR spectrophotometer for predicting alpha-amylase activities in individual Canadian Western Red Spring (CWRS) wheat kernels. Biosyst. Eng. 108, 303–310.

Yadgary, L., Cahaner, A., Kedar, O., & Uni, Z. 2010. Yolk sac nutrient composition and fat uptake in late-term embryos in eggs from young and old broiler breeder hens. Poult. Sci. 89, 2441–2452.

Zhang, W., Pan, L., Tu, S., Zhan, G., & Tu, K. 2015. Non-destructive internal quality assessment of eggs using a synthesis of hyperspectral imaging and multivariate analysis. J. Food Eng. 157, 41–48.

Zhao, J., Lin, H., Chen, Q., Huang, X., Sun, Z., & Zhou, F. 2010. Identification of egg's freshness using NIR and support vector data description. J. Food Eng. 98, 408–414.

Zhao, Q., Lv, X., Jia, Y., Chen, Y., Xu, G., & Qu, L. 2018. Rapid determination of the fat, moisture, and protein contents in homogenized chicken eggs based on near-infrared reflectance spectroscopy. Poult. Sci. 97, 2239–2245.

Chapter 6
General conclusion

The aim of the study was to investigate the effect of hen age and storage time on certain egg quality parameters, as well as the use of near infrared (NIR) hyperspectral imaging as an alternative, rapid technique to analyse quality and proximate composition. Three trials were conducted to investigate the effect of hen age on egg quality, the effect of hen age along with increased storage on egg quality and the application of NIR hyperspectral imaging for the differentiation as well as quantification of hen egg quality and chemical parameters.

In Chapter 3, it was concluded that hen age had a significant effect on most of the internal and external egg quality parameters, as well as the proximate parameters. These parameters included egg weight, egg height, egg diameter, shell thickness, shell weight, shell bumps, yolk height, thick albumen height, thin albumen height, yolk weight, albumen weight, Haugh unit (HU), thin albumen spreading, yolk colour L*, yolk colour a* and Roche yolk colour fan. However, hen age had no significant effect on thick albumen spreading, yolk colour b*, vitelline membrane integrity, blood spots and meat spots. Hen age significantly influenced the proximate parameters, which included the moisture, protein and lipid content but had no effect on the ash content. The most pronounced differences, in the majority of the parameters, were observed for eggs collected from younger hens (21 and 29 weeks of age), which was ascribed to hens reaching maturity.

A significant interaction (hen age × storage time) was observed for yolk height, HU, thick albumen height, thin albumen height, thick albumen spread, thin albumen spread, yolk colour L*, yolk colour b* and Roche colour fan (as seen in Chapter 4). On the other hand, egg weight, shell weight, shell thickness albumen weight, yolk weight, yolk colour a*, vitelline membrane integrity, moisture content and protein content were not influenced by the interaction, but were significantly influenced by the main effect, storage time. Egg height, egg diameter, shell bumps, blood spots, meat spots, lipid content and ash content were not influenced by the interaction of hen age and storage time, nor the main effect (storage time). It was concluded that the most pronounced differences occurred within the first 15 days of storage for the majority of the parameters.

The use of principal component analysis (PCA) aided in the unsupervised classification NIR hyperspectral images of eggs laid by different aged hens and stored for different time periods, respectively (Chapter 5). Principal component analysis with the aid of the score plots showed successful separation between eggs laid by younger hen ages (21, 29 and 37 weeks) and older hen ages (69 and 77 weeks) along PC1. The PC1 loadings plot revealed four distinct absorption bands, which were ascribed to protein, carbohydrates, moisture and lipids, respectively. The score and loadings plot indicated that eggs laid by younger hens had a higher protein and moisture content with lower lipid and carbohydrate content, while the opposite was observed for eggs laid by older hen ages. This trend was only confirmed for the moisture and lipid content by investigating the results

from the proximate analysis. A second PCA, showed separation in the score plot between eggs stored for shorter (0 and 15 days) and longer storage periods (45, 60 and 90 days). This separation was along PC1 and the loadings plot revealed three distinct absorption bands which was ascribed to protein, moisture and lipid content, respectively. Eggs stored for shorter periods displayed higher moisture and protein content with lower lipid content, while the opposite was observed for eggs stored for longer durations. The results obtained by the PCA was confirmed by the proximate analysis, except for the protein content which did not follow this trend.

Subsequently, the viability of NIR hyperspectral imaging in combination with partial least squares regression (PLSR) to quantitatively predict HU, protein, lipid and moisture content, as influenced by hen age as well as storage time, was also evaluated (Chapter 5). The raw NIR data obtained from imaging was preprocessed with various techniques to optimize the model conditions during cross validation. Several steps were implemented to improve models. Firstly, models constructed with the whole egg image spectra were compared to models constructed with spectra consisting of a smaller region of interest. Results showed that all models constructed with the whole egg spectra gave more favourable results. Secondly, the full spectral range was reduced to investigate if the exclusion of the end regions, which was considered to contain noisy spectra, would improve the models. It could be concluded that the reduction improved the models. However, although the models might not be excellent for prediction, it could still be used for approximate prediction and to distinguish between samples with high and low HU, protein, lipid and moisture content, respectively. The HU model gave the most favourable results, while protein, lipid and moisture gave less accurate models.

In this study, it was demonstrated that increasing hen age and storage time had a substantial, mostly negative impact on the quality of eggs, which introduces the challenge to regulate and ensure optimal egg quality. There is thus a dire need for a rapid, non-destructive and cost-effective screening method that can replace the current tedious conventional egg quality testing methods, to ensure egg safety along with quality. Near infrared hyperspectral imaging has shown great promise in the current study and for future research. The work done in the current study should be expanded by reducing the storage interval, for example to 30 days and increasing the frequency of analysis, for there was already a substantial change within the first 15 days of storage in most egg quality parameters. Models constructed based on shorter storage intervals might be of more value to the egg industry as most eggs are sold as quickly as possible after they have been laid. Fewer eggs with lower HU's were used in the model as the yolks of eggs stored for extended periods of time ruptured and could not be measured. The use of a shorter storage time interval and a reduced HU range may be more beneficial for improving the models. Future studies could investigate differences in egg quality as effected by temperature as well as humidity and if it is possible to distinguish between these samples with aid of PCA. It would also be interesting to see how accurate constructed PLS models could predict egg quality as affected by temperature and humidity. Research should also be extended to investigating structural changes in proteins, to acquire a better understanding

of how changes in proteins could affect the quality of eggs during storage. It would also be interesting to investigate the possibility to differentiate between egg quality in terms of breed differences with the aid of hyperspectral imaging and MIA. It might also be beneficial for future studies to expand MIA by exploring other analysis techniques such as partial least squares discriminant analysis (PLS-DA) or soft independent modelling of class analogy (SIMCA) that could possibly improve the differentiation between eggs of different qualities.

Near infrared hyperspectral imaging has shown great potential in the current study, offering rapid quantification of egg quality as well as differentiation between samples. Therefore, applying this technology in the egg industry could greatly add to securing eggs safe for consumption and ensure optimal quality.